TARGETING HOXB13-OVEREXPRESSING TAMOXIFEN-RESISTANT BREAST CANCER

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ABSTRACT

Aberrant expression of the homeobox (HOX) gene family of transcription factors has been implicated in the development of many different cancers, including breast. The high ratio of homeobox 13 (HOXB13) to interleukin-17-β receptor (IL17BR) expression has been found to be associated with increased relapse, tumor aggressiveness, tamoxifen (TAM) treatment failure, and death in breast cancer patients. For nearly a decade, HOXB13 has been known as an effective biomarker of TAM resistance (TAMR), however its role in the disease was unclear.

Our recent studies established that HOXB13 overexpression promotes TAMR as well as stromal invasion and metastasis via interleukin-6 (IL6) upregulation due to increased signaling of its pathway and subsequent stromal-tumor crosstalk. In order to identify other potential, targetable mechanisms of HOXB13-mediated TAMR, we used a two-pronged approach to our goal, the first, using microarray analysis to discover new targets associated with HOXB13-expression in our cell line models, and the second, to interrogate other aspects of the IL-6 pathway that can possibly be more accessible, more effective targets to inhibit HOXB13-mediated-TAMR.

Although our microarray studies did not yield novel targets of HOXB13-expressing TAMR, investigating other aspects of IL6 action in our HOXB13-expressing cell line models via inhibition of these downstream effectors found the anti-IL6R antibody tocilizumab (TOC) to specifically inhibit HOXB13-expressing tumor growth both *in vitro* and *in vivo*, as well as abrogate the stromal desmoplastic response typical of our HOXB13-overexpressing xenografts and of aggressive tumors.

These data suggest TOC to be a promising therapy able to specifically target HOXB13-expressing TAMR breast cancer, leading to more effective options for the treatment of TAMR, as well as a better understanding of the function and mechanism of the role of HOXB13 in this disease.

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CHAPTER 1

Introduction

1.1 – Breast cancer and endocrine treatment

Breast cancer is the second most common cancer among women in the United States, affecting one out of eight females (1) during their lifetime. In 2013, an estimated 232,340 new cases of invasive breast cancer were diagnosed, and during this time, almost 40,000 died from this disease (2). The incidence of breast cancer has been found to increase exponentially with age, until the onset of menopause (3).

Development of this disease usually begins in the cells of either the lobules (milk-producing glands), the ducts (tubes that branch from the lobules through which milk flows from the lobule to the nipple, or in rare cases, the stroma or connective tissue, of the breast (Figure 1.1). As with many other cancers, breast cancer develops along a continuum of stages: from hyperplasia to atypical ductal hyperplasia (4), precancerous lesions characterized by abnormal cell layers in the ducts or lobules, to ductal carcinoma in situ (DCIS), non-invasive lesions containing abnormal cells, to malignant or invasive breast cancer (IBC), where cancerous cells invade the surrounding tissue and spread to the lymph nodes or other organs (5). In addition to the different stages, grades, and types of breast cancer, the expression levels of estrogen receptor (ER), progesterone receptor (PR), ErbB2 (HER2), are also used to assess treatment options for breast cancer patients.

Estrogen is an essential hormonal regulator in normal female development and physiology, including that of the mammary glands. In pre-menopausal women, estrogen is synthesized primarily in the ovaries, whereas in post-menopausal women, the main source of estrogen the adipose tissue. The effects of estrogen on the cellular processes of proliferation and survival in the breast are mediated through the activation of the estrogen receptors ERα

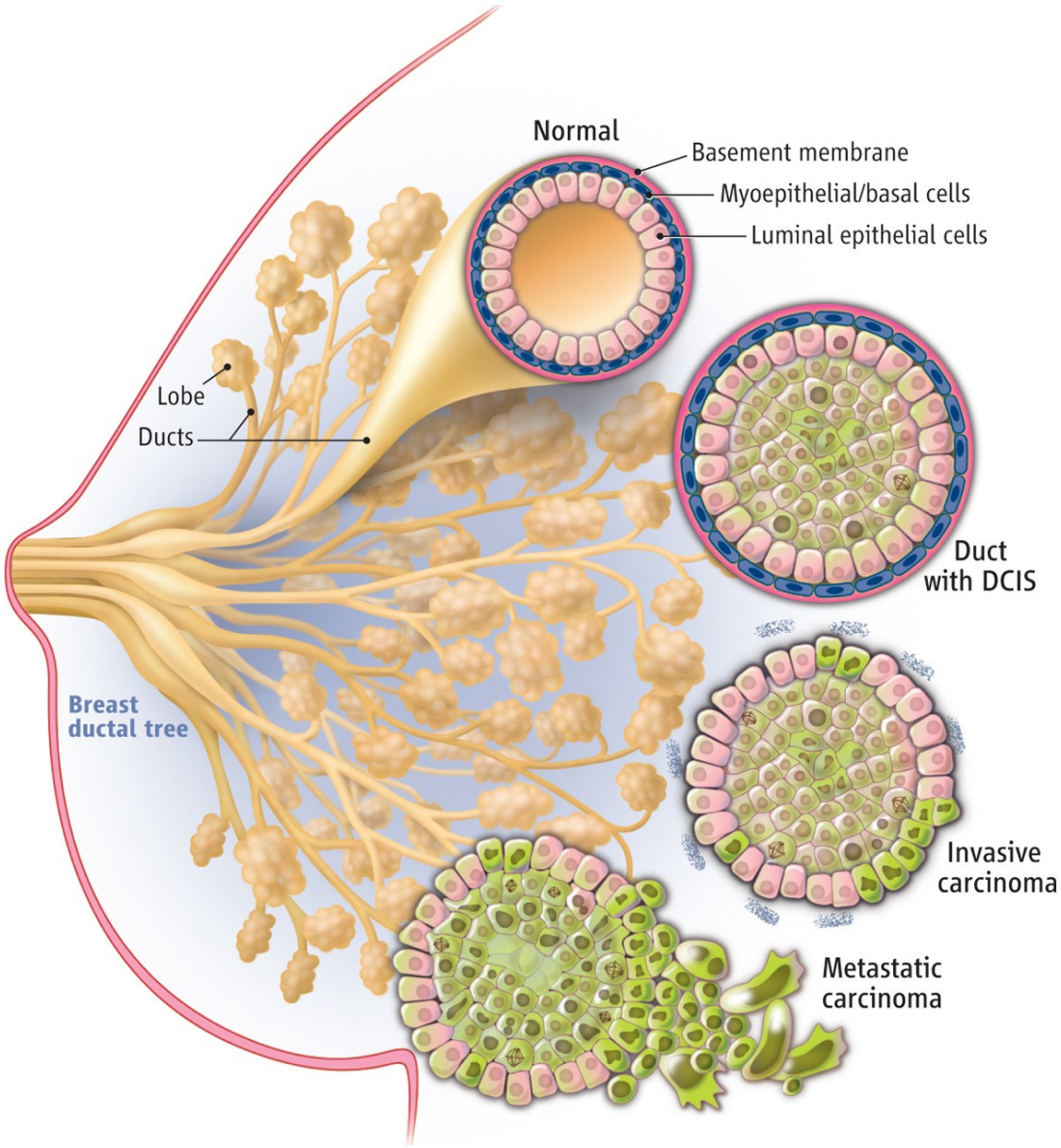

Figure 1.1. Stages of breast cancer progression (6).

(7) and ERβ (8), nuclear hormone receptors encoded by separate genes located on different chromosomes. Both receptors share similar homology in their ligand- and DNA-binding domains, as well as the ability to function as transcription factors to control the expression of ER target genes (9). However, ERα is primarily expressed in the breast and female reproductive organs, whereas ERβ is more widely distributed (10). Due to its predominant expression in the breast, ERα is responsible for most of the estrogenic effects on both normal and oncogenic breast tissue through either genomic (Figure 1.3a) or non-genomic mechanisms (Figure 1.3b).

Genomic (classical) ERα signaling occurs upon estrogen binding of ERα, causing a conformational change in the receptor, ERα phosphorylation, dimerization, and activation, where ligand-bound-ERα binds cofactors, such as coactivators AF1 and AF2 (Figure 1.4). This ligand-receptor-cofactor complex then undergoes a conformational change and collectively binds estrogen response elements (EREs), specific DNA sequences in the promoter regions of target genes, to control ER target gene transcription (11). (Non-classical) ERα signaling may also occur via ligand-bound-ERα formation of a complex with cofactors Fos and Jun to bind AP-1 binding sites, activating target genes lacking EREs. Genomic ERα activation may occur not only by ligand-receptor interaction, but also via growth factor membrane receptor signaling such as through the IGF1 or EGFR families, by direct phosphorylation of ERα or its cofactors (12). Non-genomic ERα activation occurs outside the nucleus where cytoplasmic ERα interacts with various intracellular signaling pathways such as the cyclic AMP/cAMP-responsive-element-binding protein (CREB), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) pathways, thus affecting the transcription of other target genes (13).

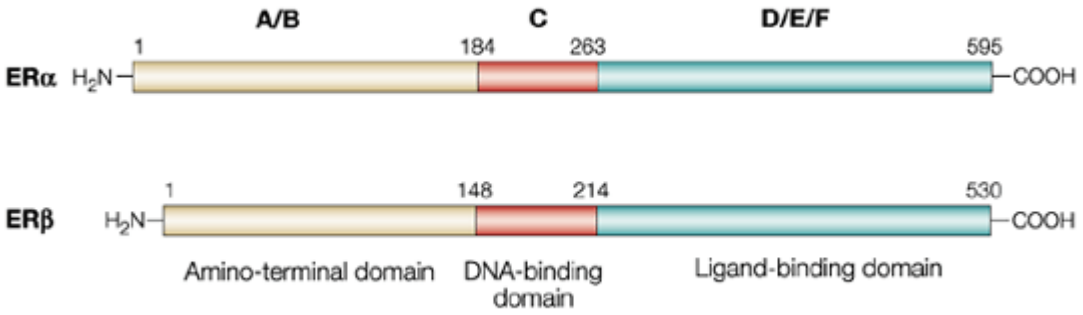

Figure 1.2. Domain structures of ERα and ERβ. Both human ERα and ERβ share structural domains (A-F). From a functional perspective, ERs are divided into three parts: the amino-terminal or A/B domain, the DNA-binding or C domain, and the ligand-binding or E domain. The F domain is involved in distinguishing between estrogen agonists and antagonists (13).

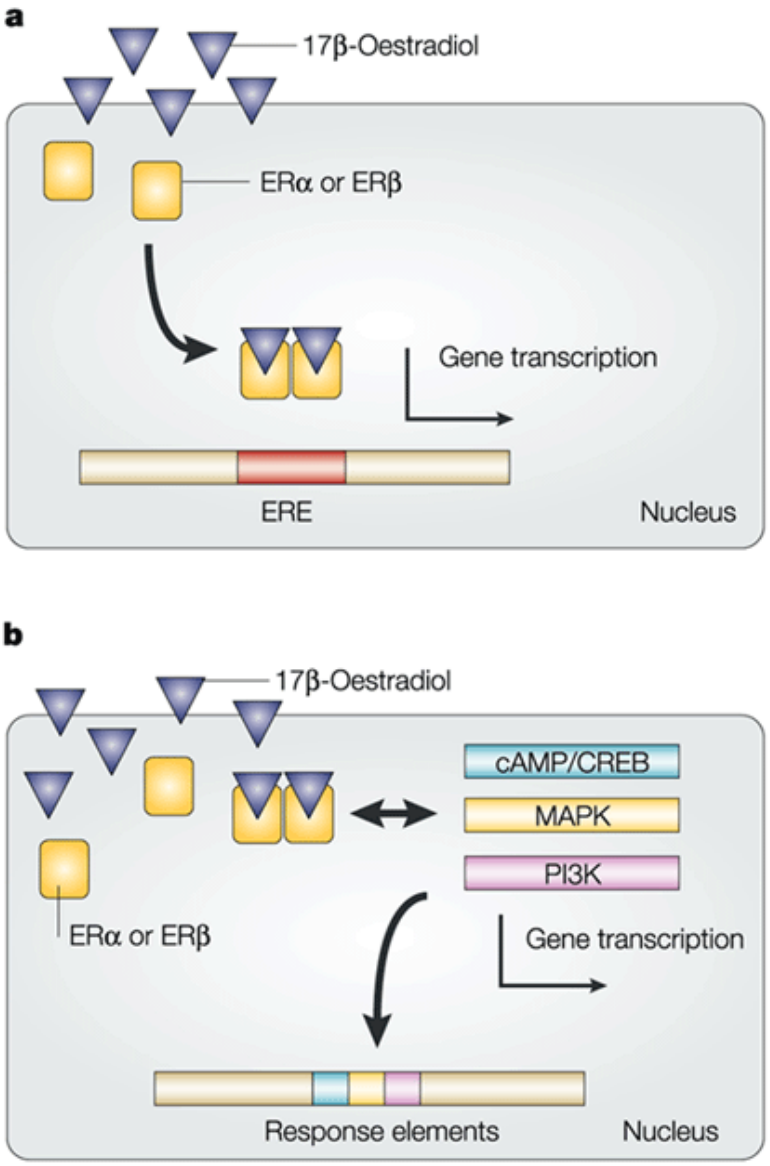

Figure 1.3. Simplified model of the genomic and non-genomic modes of estrogen action. A. The classical mode of action of ERs involves ligand binding to the cytoplasmic receptor, translocation to the nucleus and binding to estrogen response elements (EREs) in gene promoters, resulting in the induction of gene transcription. Once bound to the ERE, further factors (not shown), such as AF1 and AF2, as well as co-activators, are involved in the induction of gene transcription. Last, transcription factors and RNA polymerase complement the transcription machinery. B. In addition to this genomic mode of action, ERs might interact directly with various intracellular signalling pathways, including the cyclic AMP/cAMP-responsive-element-binding protein (CREB), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, thereby indirectly affecting the transcription of several other target genes (13).

At least 70% of breast cancers are identified as ER-positive in that their growth is dependent on estrogen. Due to this fact, interference with estrogen action has been an essential means of breast cancer treatment as early as 1896, where Beatson reported that surgical removal of the ovaries from advanced breast cancer patients led to tumor regression and improved diagnosis (14). Although oophorectomy and eventually ovarian irradiation became standard for pre-menopausal patients into the 1960s, only one-third of patients responded to such treatments (15). In the 1970s, development of the estrogen-ER binding theory with regard to estrogen action in breast cancer led to the synthesis of competitive inhibitors against estrogen binding to ER and the development of the most established and widely used selective estrogen receptor modulator (SERM), tamoxifen (16). After tamoxifen, other drugs were developed as alternative endocrine therapies, such as fulvestrant, whose mode of action is to bind, block, and lead to ubiquitination and proteosomal degradation of ER, inhibiting ER signaling (17), as well as aromatase inhibitors (18), which inhibit estrogen synthesis, and whose third-generation non-steroidal AIs letrozole and anastrozole and steroidal AI exemestane (19).

TAM is a non-steroidal triphenylene derivative and works as a competitive inhibitor against estrogen to bind ER. Upon TAM binding to ER, the receptor dimerizes and binds DNA, where, in contrast to estrogen, TAM blocks the transcriptional activating function of AF2, as AF2 activation is hormone-dependent, and therefore inhibits activation of AF2 ER-target genes, impeding breast cancer cell survival and proliferation. The coactivator AF1 does not require ligand-binding and thus, retains its activity upon TAM binding, transcribing ER target genes that require AF1 action (Figure 1.4).

In spite of the partial activation of AF1 ER target genes, TAM has been highly effective as first-line adjuvant therapy for post-menopausal women with early-stage ER-

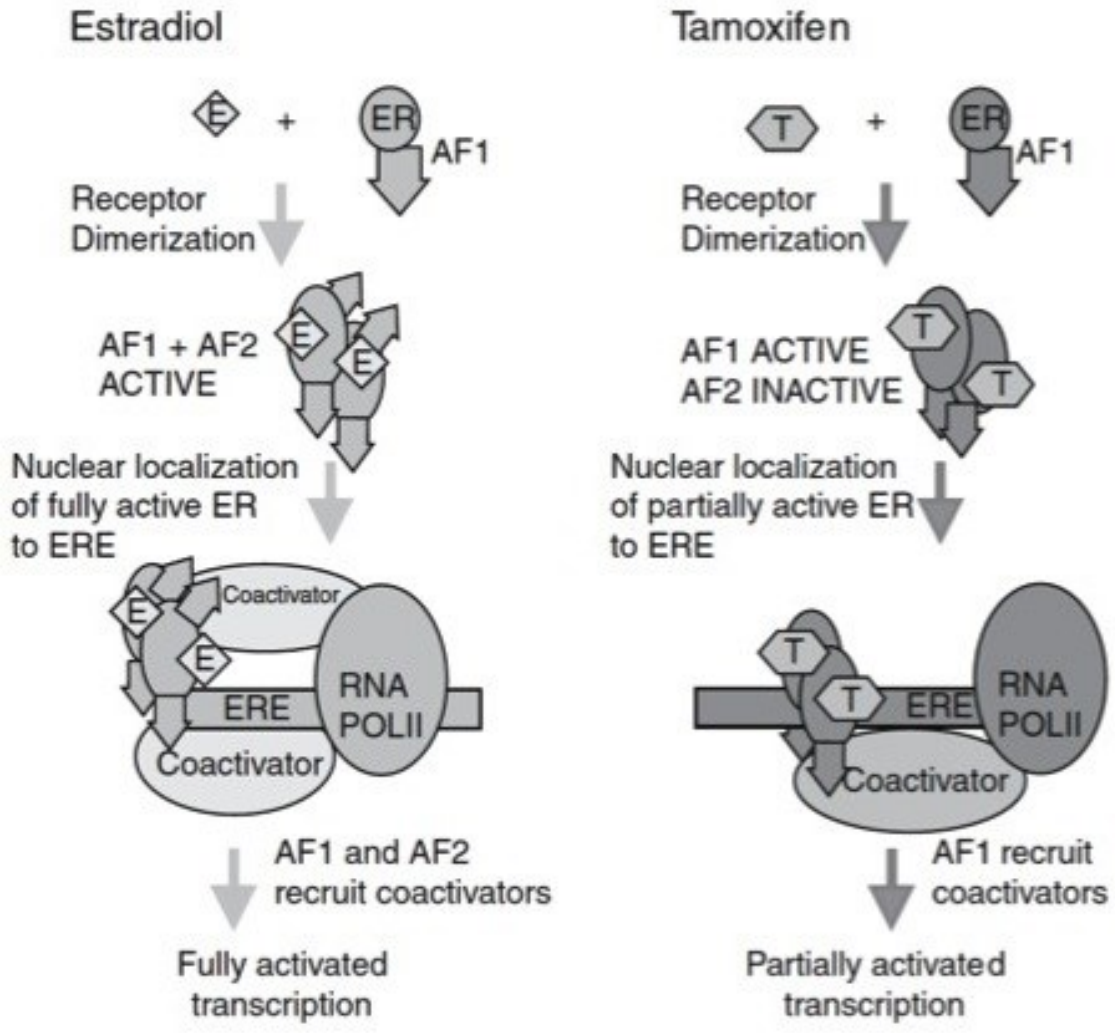

Figure 1.4. Mechanism of action of estradiol/estrogen (E) compared to tamoxifen (T) at the level of transcriptional regulation. E binds to ER, leading to dimerization, conformational change and binding to estrogen response elements. T competes with E for ER binding to inhibit estrogen signaling (20).

positive breast cancer, almost halving the rate of disease recurrence and reducing the annual breast cancer death rate by one-third. Unfortunately, 30-40% of breast cancer patients treated with TAM over 5 years eventually relapse within 15 years with distant metastasis, most succumbing to their disease (21). Median overall survival in these patients, even with further treatment, is around 30 to 45 months (22).

1.2 – Mechanisms of tamoxifen resistance (TAMR)

Extensive studies have identified multiple mechanisms by which breast cancers exhibit tamoxifen resistance (TAMR), classified into two categories: intrinsic (de novo) or acquired. Intrinsic TAMR breast cancers are predisposed to be unresponsive to TAM due to mutations in the ER ligand binding domain which stabilize ER, rendering it constitutively active (23, 24), the lack of ER α expression also via mutation, in this case, rendering the tumors functionally ER-negative, inactivation via methylation, truncated ER isoforms, as well as post-translational modifications (25). Another mechanism of intrinsic TAMR includes expression of the inactive alleles of the drug-metabolizing enzyme cytochrome P450 2D6, which fail to convert TAM to its active metabolite endoxifen, rendering tumors less responsive to TAM (26).

Acquired TAMR in breast cancer is characterized by initial response to TAM, which, over time, results in eventual relapse and progression. TAMR occurs through multiple mechanisms, including modulation of the ER pathway, such as through increased activity of the AP1 and NFκB co-activating transcription factors, deregulation of compensatory cellular signaling pathways, such as through the MAPK and PI3K/Akt pathways, as well as the disruption of cell cycle and apoptosis processes (25). Although many molecular events that confer TAMR are known, a unifying theme is still unknown.

1.3 – HOX genes

The HOX family of homeobox genes is a group of transcription factors essential for normal embryonic development, morphogenesis, and cell differentiation highly conserved across species. HOX genes collectively contain a common 120-base pair homeobox DNA sequence element encoding the 61-amino acid peptide called the homeodomain, which binds the consensus core DNA sequence 5'-TAAT-3' (27). HOX genes were first described in the fruit fly Drosophila melanogaster as the genes responsible for proper spatial body development (28). A series of duplication events throughout evolution is thought to have given rise to homologs of these genes throughout vertebrates, and eventually to the 39 HOX genes found in humans. Human HOX genes are organized into 4 paralogous clusters named A, B, C, and D, where each cluster is localized on a different chromosome and may contain from 9 to 13 genes numbered 1-13 according to their 3' to 5' order of alignment (29). The HOX gene family members function as transcription factors, acting as monomers or heterodimers with the three-amino-acid-loop extension or TALE cofactors; however, due to their evolutionary duplication and clustering across species, HOX genes in higher, more complex animals exhibit functional redundancy, notably across the paralogs.

The HOX genes follow a specific spatio-temporal colinearity or pattern of expression that reflects their genomic order in their respective clusters, where the 3' to 5' organization of the HOX genes on the chromosome corresponds to the order and timing of their expression along the anterior-posterior axis of the developing animal (Figure 1.5). HOX genes also exhibit posterior prevalence, where HOX genes located more 5' within the cluster will have a more dominant phenotype to those located more 3' (30).

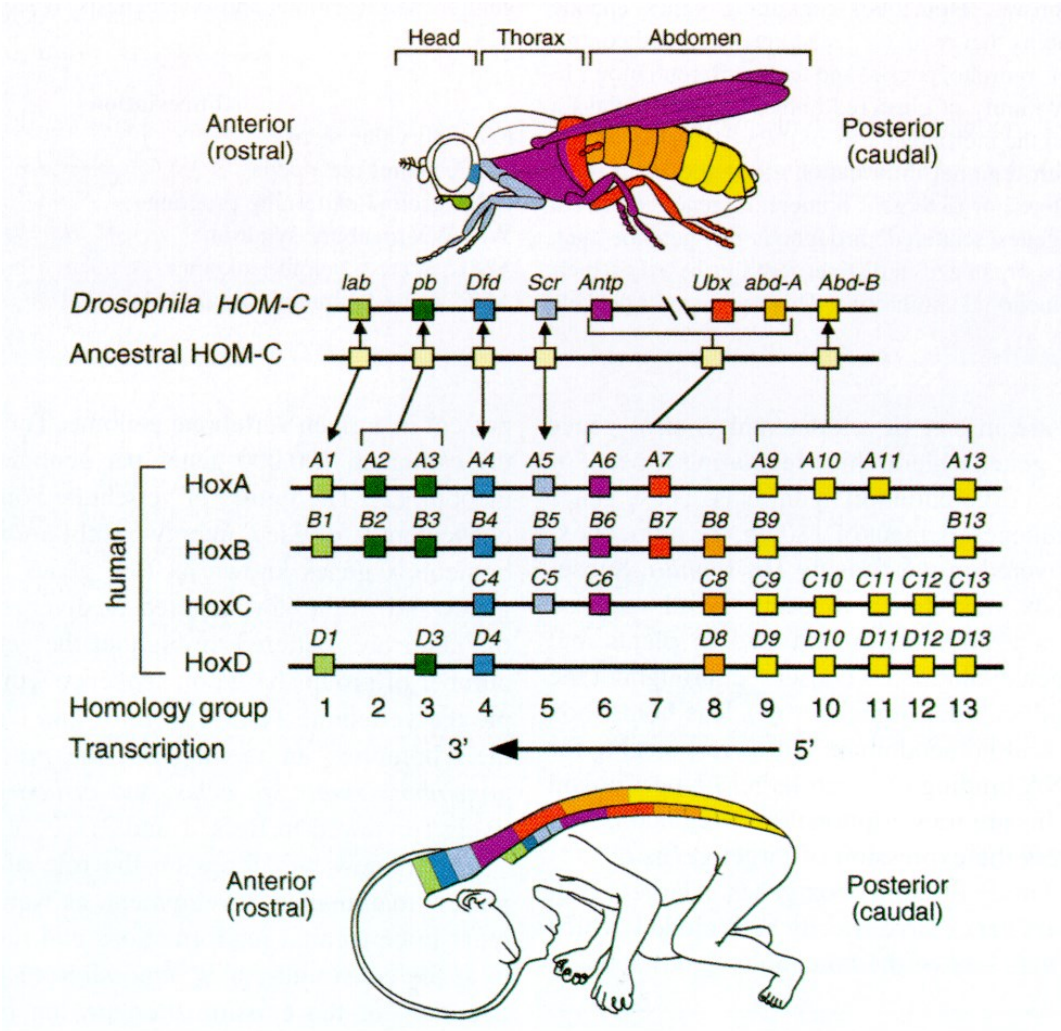

Figure 1.5. Genomic organization and colinear expression patterns of Drosophila HOM and Human HOX genes (30).

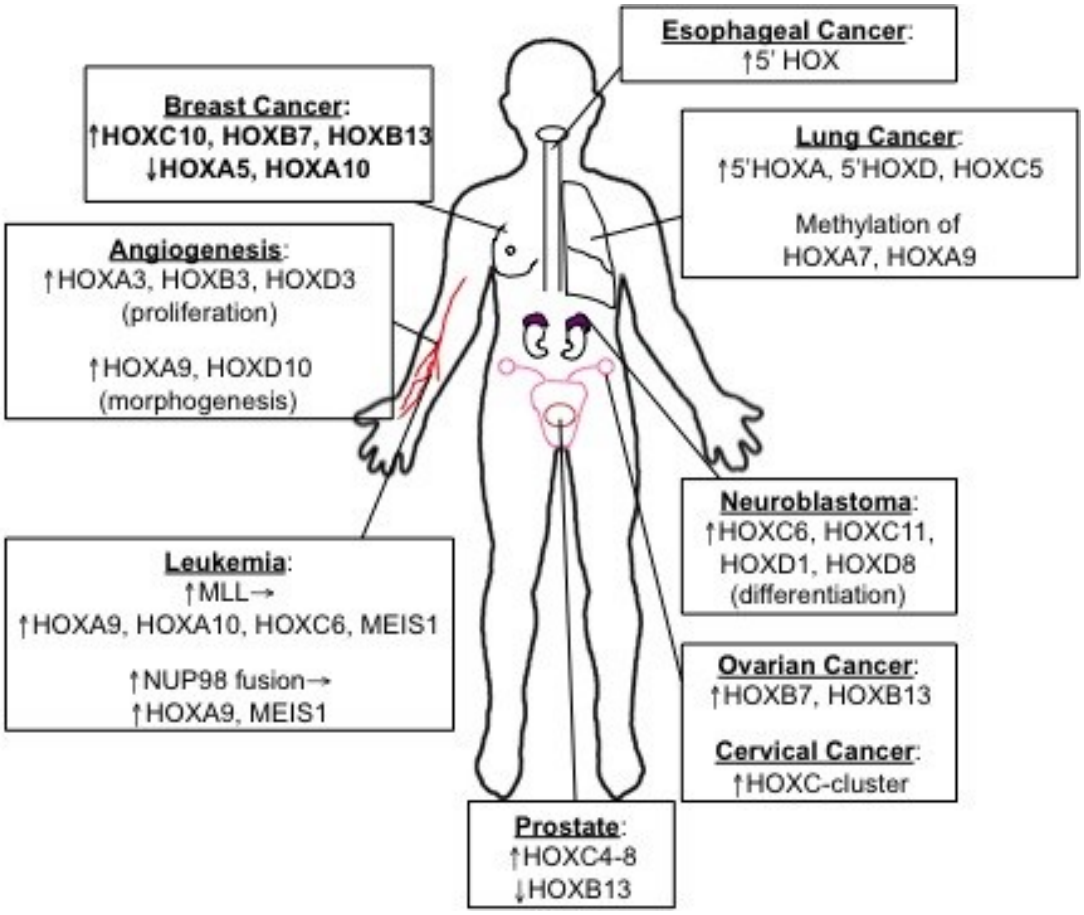

Figure 1.6. Human HOX gene deregulation in cancer (Nilay Shah).

HOX genes function as transcription factors that regulate essential processes in the body, such as cell differentiation and proliferation in embryonic development, as well as cell motility and apoptosis, important functions in adult tissue (29). HOX gene expression is frequently deregulated in numerous types of cancer (Figure 1.6). In breast cancer, the loss of HOXA5 and HOXA10 expression has been associated with high-grade breast tumors, leading to decreased apoptosis and p53 expression (31, 32), whereas overexpression of HOXB7 and HOXB13 has been shown to play respective roles in aggressive disease and TAMR (33, 34).

1.4 – HOXB13 and TAMR

HOXB13 was the last human HOX gene to be identified, located about 70 kilobases upstream of the HOXB cluster on chromosome 17. HOXB13 is normally expressed in the posterior body axis, including the spinal cord, urogenital tract, colon, and prostate, functioning as a regulator of terminal cellular differentiation (35). Loss-of-function mutations in HOXB13 in mice result in the structural overgrowth of major tissues derived from the tail-bud, as well as decreased apoptosis in the secondary neural tube during development (36).

Aberrant expression of HOXB13 is associated with a number of different cancers. With regard to prostate cancer, where HOXB13 is expressed in the normal prostate and is vital to the normal development of this tissue, downregulation of HOXB13 has been shown to cause loss of differentiation as well as the transactivation of AR and cell proliferation in prostate cancer (37, 38). Also, the recurrent HOXB13 G84E mutation in prostate cancer has been found to be a prostate cancer susceptibility allele, associated with early-onset and hereditary disease (39). Downregulation of HOXB13 has also been shown to induce tumorigenesis in colon cancer (40). In tissues where its expression is normally absent,

upregulation of HOXB13 has been shown to cause invasion potential in endometrial cancer (41) and to cause tumorigenesis and abrogation of the antagonistic effects of TAM in ovarian cancer (42).

Seminal studies regarding aberrant HOXB13 expression in breast cancer highlighted the ratio of high HOXB13 to low interleukin-17-β receptor (IL17BR) mRNA expression as a marker of predictive outcome via gene expression profile analysis of hormone-positive primary breast cancer in patients treated with adjuvant TAM mono-therapy (43). A high ratio of HOXB13/IL17BR (H:I) was associated with increased relapse, tumor aggressiveness, TAM treatment failure, and death in breast cancer patients (43, 44). Breast cancers in TAMR patients were also found to exhibit high HOXB13 expression, where HOXB13 overexpression was shown to be repressed by estrogen and this repression was inhibited in the presence of TAM. The Breast Cancer Index or BCI was developed as a combination of the H:I ratio with the molecular grade index of MGI of 5 other TAMR biomarkers (45), shown to predict long-term recurrence risk after 5 years of not only TAM but also AI treatment, making this biomarker relevant to the clinical management of breast cancer (46).

For nearly a decade, HOXB13 was known as an important biomarker of TAMR, but its role in the disease was unclear. Our recent studies provided some insight into the mechanism whereby HOXB13 mediates TAMR and metastasis. We showed that TAMR and metastasis could be mediated via induction of IL6 expression and subsequent activation of its signaling pathway (33) and have now performed further work into targeting aspects of the IL6 pathway as a means of treatment. This work shows that targeting the IL6 receptor (IL6R) using tocilizumab, an anti-IL6R antibody, is an effective treatment against the growth of HOXB13-overexpressing, TAMR cell line models. Thus, tocilizumab may prove to be a

novel and promising drug candidate for the therapy of HOXB13-overexpressing TAMR breast cancer.

CHAPTER 2

Materials and Methods

Human tissue specimens. Normal breast epithelial preparations (organoids), primary breast tumors, and distant metastases were accessed with approval from the Johns Hopkins University Institutional Review Board, and RNA extracted as previously described (47). Briefly, tissues from reduction mammoplasty performed at Johns Hopkins Hospital were mechanically macerated, and then digested overnight with hyaluronic acid and collagenase. The terminal ductal units were placed into suspension, and then isolated by serial filtration. Samples were treated with TRIzol and RNA extracted. Fresh frozen primary breast tumors were obtained from the Department of Surgical Pathology tumor bank; specimens were from patients 45-55 years of age with localized disease, with positive estrogen receptor expression by immunohistochemistry as performed during routine staging at diagnosis, for uniformity of samples. Metastatic breast carcinoma samples were obtained from the Rapid Autopsy Program at Johns Hopkins Hospital. All specimens were fast frozen at autopsy and stored at -80°C. Twenty 20-micron-thick sections were obtained from tumor metastases and macerated with the BioMasher sample preparation device (Cartagen), with 350 μL of lysis buffer from the Qiagen RNEasy Mini Extraction kit. RNA extraction was completed with the flow-through from the BioMasher, as per the manufacturer protocol. 10 micron-thick H&E-stained tissue sections were made from all tumor samples and were confirmed to have at least 70% non-necrotic neoplastic epithelial cellularity before RNA extraction.

Cell culture, plasmids, and cell line constructs. The breast cell lines MCF7 and BT474 were provided by NCI (IBC-45 panel) through the American Type Culture Collection (ATCC) and the human mammary fibroblast cell line 82-6 was provided by Judith Campisi

(48). MCF7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gemini Bio-Products), BT474 cells were grown in RPMI media with 10% FBS, and 82-6 cells were grown in DMEM F-12 50/50 with 10% FBS. Plasmids containing the full length cDNA of human HOXB13 in the pLPCX retroviral vector (pHOXB13), the empty pLPCX vector (Clontech), 2 short hairpin RNA (shRNA) lentiviral constructs targeting HOXB13 mRNA (shHOXB13) and scrambled shRNA construct (PLKO.1/Thermo Scientific) were used to generate viral supernatant for overexpression or knockdown of HOXB13 in cell lines (33).

Plasmid Constructs, Viral Packaging, and Cell infection. A plasmid containing the full length cDNA of human HOXB13 in the pDNR-LIB vector (catalog number MHS1011-62759), a set of short-hairpin RNA microRNA (shRNAmir) lentiviral constructs targeted against the HOXB13 mRNA (RHS4533), and a non-silencing lentiviral shRNAmir (RHS4346) were purchased from Open Biosystems. The HOXB13-pDNR-LIB vector was digested with the restriction endonuclease Sfi1 and the HOXB13 cDNA ligated into the retroviral vector pLPCX (Clontech) with T4 DNA ligase. The plasmid was transformed into Turbocells competent E. coli (Genlantis) per manufacturer protocol, which was then selected on LB agar supplemented with ampicillin (100 μg/mL). The resultant plasmid and the shRNAmir plasmids were amplified in LB broth with ampicillin and purified with the QuantumPrep Plasmid Midiprep kit (Bio-Rad). Sequences were confirmed with the Applied Biosystems 3730xl DNA Analyzer. The plasmids were then co-transfected with pCL-Ampho packaging vector (pLPCX) or psPAX2 and pMD2.G packaging vectors (lentiviral plasmids) into HEK-293T cells with Lipofectamine 2000 (Life Technologies), and viral supernatant collected at 36 and 72 hours. MCF7 and T47D cells were treated with DEAE-

dextran-containing media, then the HOXB13-pLPCX or pLPCX-vector control viral supernatant for 24 hours. Infected cells were re-plated and treated with media containing puromycin, 0.2 μg/mL, for 2 weeks. Clones were selected and HOXB13 expression evaluated. The BT474 cell line was treated with DEAE-dextran containing media, then viral supernatant from either the non-silencing shRNAmir (Scramble or Scr) or constructs against HOXB13 for 24 hours. The cell lines were re-plated and treated with media containing puromycin, 0.1 μg/mL for 2 weeks. The propagated colonies were then re-infected with either shScramble, or HOXB13-targeting shRNAmir clones TRCN0000020844 (shHOXB13-1) or TRCN0000020845 (shHOXB13-2), and infected cells reselected with puromycin 0.2 μg/mL. The resultant clones (BT474-Scr, BT474 B13 KD-1, and BT474 B13 KD-2) were then tested to confirm loss of HOXB13 expression. All cell lines were maintained in media supplemented with puromycin 0.2 μg/mL. Experiments were performed in media without puromycin.

Promoter-lucifrase reporter assay. MCF7 cells were transiently transfected with Lipofectamine 2000/DNA complexes (Life Technologies) of pGL2-HOXB13, promoter-luciferase construct (pGL2; Promega), and β-galactosidase (β-gal) plasmid, and incubated for 24 hours. Luciferase and β-gal activity were measured per protocol (Promega). Assays were conducted in triplicate in a single experiment, and then as 3 independent experiments.

Chromatin Immunoprecipitation (ChIP). ChIP was performed with the EZChip kit (Millipore) according to protocol. 2.5 x 10^6 cells were seeded into 15 cm plates and grown to 90% confluency. Protein and DNA were crosslinked with 1% formaldehyde in water and collected. Cells were sonicated with a Branson 450 ultrasonicator (4 cycles of 30 seconds

each, 30% Duty cycle, output 4) on ice. The resulting lysate was either stored as input DNA or immunoprecipated with anti-HOXB13 antibody (F-9, Santa Cruz), normal mouse IgG, or anti-RNA Polymerase II (Millipore) overnight at 4°C after pre-clearing, then complexes collected with ChIP-grade Protein G Agarose. The DNA crosslinking was reversed and DNA purified by spin column. 2 μL of the eluted DNA was then used for PCR, using primers designed to noted regions of the IL6 promoter, extending 2000 bp upstream of the transcription start site. PCR was performed using BlueTaq polymerase (Denville Scientific), using the provided buffer, per manufacturer protocol. Half of each reaction was run on 1% agarose/LB gel. Amplification of DNA in the input and anti-HOXB13 fractions and lack of amplification in the normal IgG fraction were indicative of specific binding of the probed DNA region by the HOXB13 antibody.

Microarray analysis. Gene expression microarray analysis was performed on BT474-Scr and two clones of BT474-B13 KD cells. The data was analyzed (by Sean Soonweng Cho) using the R statistical environment using packages from Bioconductor and custom functions when necessary. The data was RMA normalized and log2 converted for further analysis. Unsupervised hierarchical clustering was performed on 500 most differentially expressed genes across all three samples. Unsurprisingly, the BT474 B13 KD knockdown cell lines formed a tight cluster compared to the BT474-Scr control. To identify genes that are differentially expressed between knockdown and control cells, we compared the log2 expression of both conditions and genes with a log2 fold change of larger than 1 is considered differentially expressed.

Cell line RNA extraction and quantitative real-time PCR (qRT-PCR) validation of gene expression. Cells were grown in 6-well plates and harvested at equal density (approx. 80% confluence) with TRIzol (Life Technologies). RNA was then extracted and purified per manufacturer protocol. A total of 2 μg of RNA from cell lines was reverse-transcribed using MMLV-RT (Promega). Primers were designed using Primer3 (49) to span an intron. qRT-PCR was then performed using the Maxima SYBR Green/ROX Master Mix (Fermentas) per manufacturer protocol. Gene expression was analyzed by the ΔΔCt method, with GAPDH expression used for normalization.

Western blot analysis. Cells were grown in 6-well plates and after 24 hours, medium was changed to either complete medium with phenol red supplemented with 10% heat-inactivated fetal bovine serum, or phenol red-free medium supplemented with 5% charcoal-stripped FBS and treated with the drug specified. At the indicated time, cells were washed with PBS and lysed with RIPA buffer for 1 hour and then centrifuged for 30 minutes at 4°C. Total protein concentration was measured using the BCA Protein Assay according to the manufacturer's protocol. Equal amounts of extracted lysate were vertically electrophoresed on a 4-12% Bis-Tris NuPage Novex Gel in MOPS SDS running buffer (Life Technologies), then transferred to a Hybond C Extra membrane (GE Healthcare). Membranes were stained with Ponceau stain to confirm protein transfer, then blocked with 5% nonfat dry milk in PBS with 0.2% Tween-20 (PBST) for one hour at room temperature. Membranes were probed with primary antibody in 5% Bovine Serum Albumin (BSA)/PBST at 4°C overnight, rinsed with PBST, then probed with secondary antibody (GE Healthcare) at 1:4500 dilution in 5% milk/PBST for 1 hour. After rinsing with PBST, membranes were treated with ECL Plus Detection Reagent (GE Healthcare) for 1 minute, and exposed to

HYblot CL autoradiography film to determine protein expression. Antibody against HOXB13 was purchased from Santa Cruz; antibodies against beta-catenin, STAT3, Akt, and P70S6K were purchased from Cell Signaling; GAPDH antibody was purchased from Millipore.

MTT assay. $2.5x10^3$ cells/well were plated in 96-well plates in triplicate and treated with the indicated drug concentrations, alone, or in combination. After 48 hours, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Quality Biologicals) solution (5 μg/mL) was added, cells incubated for 3 hours, dissolved in 1:1 DMSO:EtOH, and absorbance at 560 nm was measured, with background at 670 nm subtracted. Values are expressed as percent survival of the vehicle-treated control (given as 100%).

Migration assays. Migration assays were performed in BD BiocoatTM Control (24-well format) Invasion Chambers per manufacturer protocol. 750 μL of chemoattractant was used. $8x10^3$ 82-6 Immortal Human Mammary Fibroblast cells were placed in the chamber in 500 μL serum-free media. Cells were incubated at 37°C for 24 hours, and then membranes scrubbed, fixed with 15% formalin, and stained with 1.25% crystal violet. Slides were made of the membranes and migrating cells counted. Cell-conditioned media were tested in triplicate.

Tumor xenograft studies. 6-to-8-week-old female NSG mice were used and the study approved by the Johns Hopkins Animal Use Committee. Cells were grown to 90% confluence, trypsinized, re-suspended in PBS, and mixed 1:1 with Matrigel (BD Biosciences). On day 0, $3x10^6$ cells in total volume 50 μL of MCF7 B13-2 cells were injected

subcutaneously (s.c.) into both flanks of each mouse. Upon palpable tumor, 5 mice with subcutaneous tumors were treated with (a) NVP-BEZ235 (50), 25 mg/kg administered orally (p.o.) dissolved in 10% NMP-90% PEG, freshly formulated, dose administrated within 30 minutes, (b) Tocilizumab, 100 ug intraperitoneally (i.p.) or (c) NVP-BEZ235 and Tocilizumab, or (d) no treatment. In a second experiment, 6-to-8-week-old female NSG mice were used and the study once again approved by the Johns Hopkins Animal Use Committee. Cells were grown to 90% confluence, trypsinized, resuspended in PBS, and mixed 1:1 with Matrigel (BD Biosciences). On day 0, $3x10^6$ cells in total volume 50 μL of MCF7-Vector, MCF7 B13-2, BT474 B13 KD-2, or BT474-SCR cells were injected s.c. into both flanks of each mouse. Upon reaching 100 mm^3 tumor size, 5-6 mice each with two subcutaneous tumors (10-12 tumors) were treated as above with either (e) Tocilizumab, (f) Tamoxifen (51) 500 μg administered s.c., suspended in peanut oil, freshly formulated, dose administrated within 30 minutes, or (g) Tocilizumab and Tamoxifen, or (h) no additional treatment. Tumor growth was measured about every 3 days. Mice were euthanized after 6 weeks of treatment and tumors harvested, sectioned and flash-frozen, or formalin-fixed and paraffin-embedded and H&E slides made. Tumor volume was estimated by the calculation V = (length x width x height x 0.5236) mm^3.

Statistical analyses. Results were expressed as mean ± standard error of the mean (52) of at least 3 independent experiments. All statistical tests were two sided, and differences were considered statistically significant at $P<0.05$. Analyses were performed using Microsoft Excel (2011).

CHAPTER 3

HOXB13 mediates tamoxifen resistance and invasiveness in human breast cancer by inducing IL6 expression

3.1 – HOXB13 is overexpressed in primary ER-positive breast cancers and distant metastases

In order to verify HOXB13 overexpression in breast cancer, we interrogated ER-positive breast cancer samples from breast cancer survivors, non-survivors, and metastases from multiple tissues for HOXB13 expression by qPCR. Compared to normal organoids, each group of tumors had significantly higher expression of HOXB13. Tumors from non-survivors and metastases showed even greater overexpression of HOXB13 compared to those from survivors (Figure 3.1), thus confirming HOXB13 overexpression in ER-positive breast cancers and distant metastases.

3.2 – HOXB13 overexpression promotes TAMR

Based on the above data demonstrating HOXB13 overexpression in clinical samples of ER-positive breast cancer, which was higher in non-survivors and metastases, we hypothesized that HOXB13 plays an integral role in tumor progression in patients with ER-positive breast cancer. In order to study the mechanism by which HOXB13 mediates tumor progression to TAMR and distant metastasis, we created stable clonal cell line models of HOXB13 expression; two stable clonal cell lines of HOXB13-overexpressing were made in MCF7 (MCF-7-B13-1 and -2), and reciprocally, stable clonal cell lines were also created where HOXB13 was knocked down with shRNA in the BT474 cell line (BT474-shB13-1 and -2), which exhibits endogenous high HOXB13 expression (Figure 3.2a).

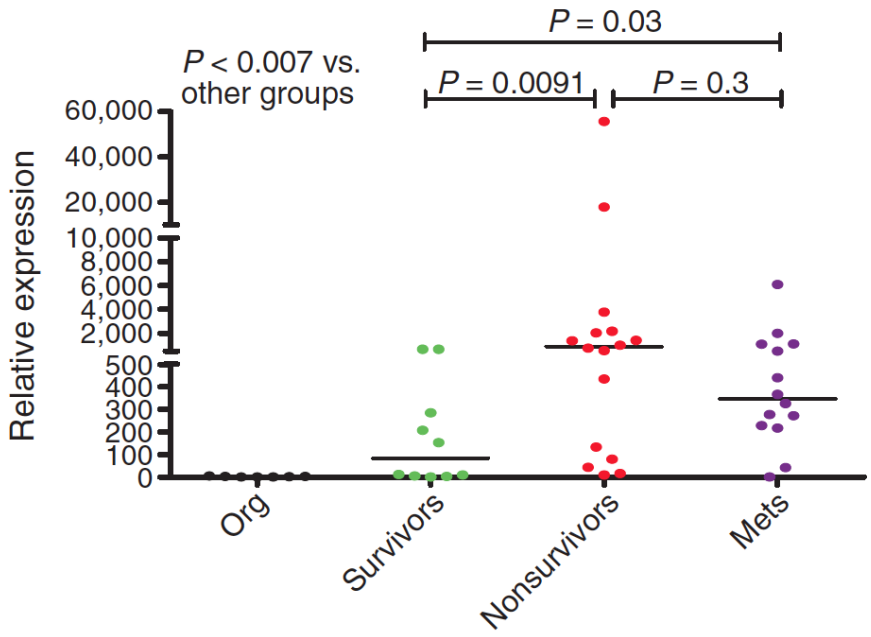

Figure 3.1. HOXB13 is overexpressed in primary ER-positive breast cancers and distant metastases. qPCR validation of HOXB13 expression in normal breast organoids (Org), ER-positive primary breast tumors from long-term survivors or non-survivors of disease, and metastases (mets). Median expression is marked.

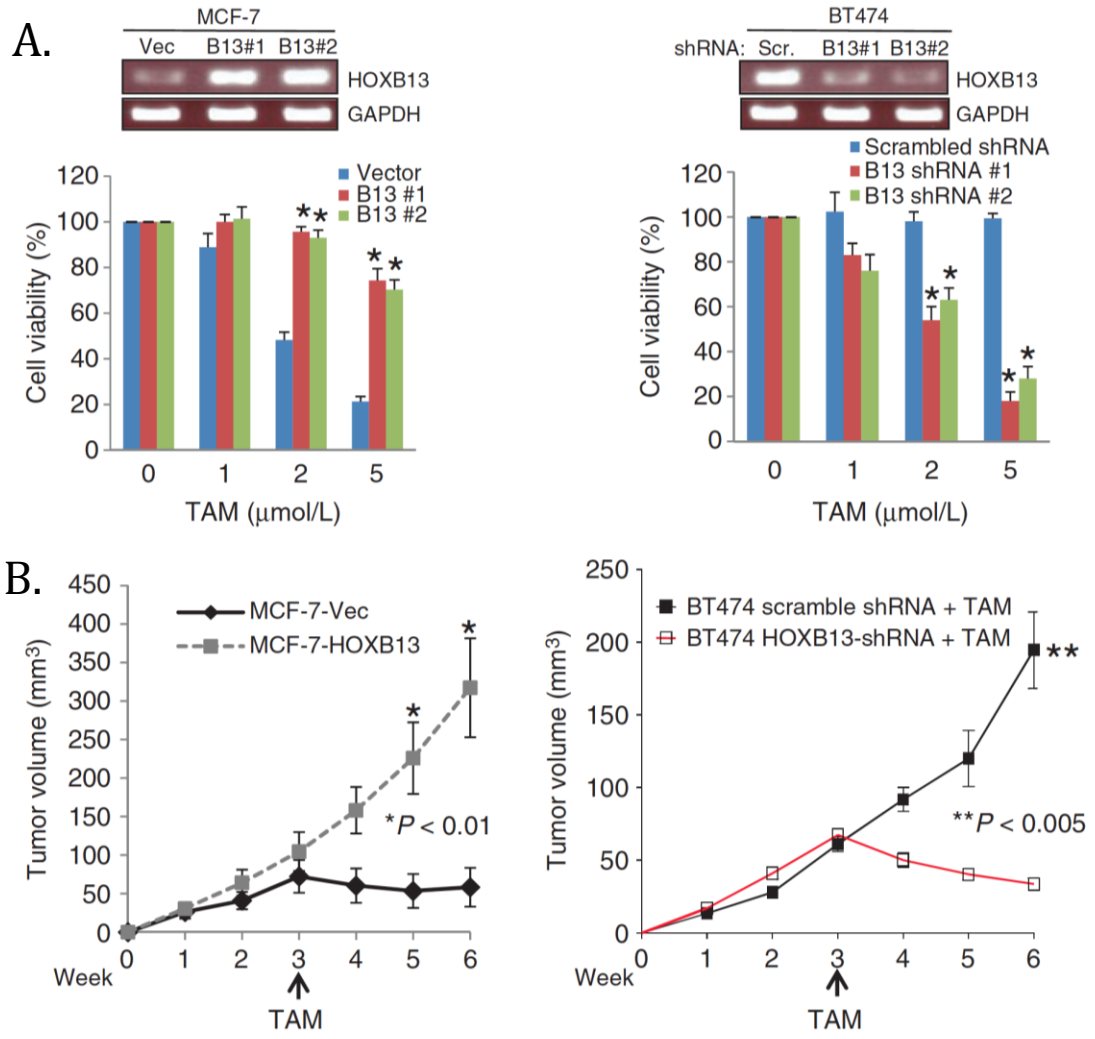

Figure 3.2. HOXB13 overexpression promotes TAMR. A, qPCR analysis of HOXB13 expression in MCF7 and BT474 HOXB13-expressing cell line panels compared with vector control; cell viability after treatment with 1, 2, or 5 mmol/L 4-OH TAM. B, tumor growth of MCF7-vector (black) and MCF7- HOXB13 cell subcutaneous xenografts in athymic mice with E2 supplementation, then treated with subcutaneous TAM implant (arrow; *, P<0.01); tumor growth of BT474-scrambled shRNA (closed square) and BT474- HOXB13 shRNA (open square) cell subcutaneously xenografts in athymic mice with E2 supplementation, then treated with subcutaneously TAM implant (**, P < 0.005).

To determine if HOXB13 overexpression correlated with TAMR, we treated MCF-7-HOXB13 clones with increasing concentrations (1 to 5 μM) of 4-hydroxytamoxifen (4-OH TAM, the active metabolite of TAM) for 48 hours and tested for cell viability. The HOXB13-overexpressing cells showed higher cell survival to TAM treatment at higher concentrations as opposed to vector control in MCF7 and in BT474, the HOXB13-expressing BT474-Scr cell line also showed higher cell survival to TAM treatment, whereas HOXB13 knockdown rendered these BT474 clones sensitive to TAM, decreasing their survival (Figure 3.2a). Growth phenotypes of these cells were also tested *in vivo*. TAM treatment of MCF7 vector control xenografts resulted in regression of the tumors, whereas TAM treatment did not have a significant effect on the growth of MCF7 HOXB13-overexpressing xenografts which continued to grow despite TAM (Figure 3.2b). HOXB13-expressing BT474-Scr tumors also continued to grow upon TAM treatment, whereas the BT474 HOXB13 knockdown tumors regressed. Thus, MCF-7 respond to tamoxifen, while high HOXB13 expressing MCF-7-HOXB13 cells do not. Conversely, BT47scr cells are high HOXB13 expressors, and are resistant to the effects of TAM, while the HOXB143-KD cells are susceptible to tamoxifen. Collectively, these data reflect the TAMR phenotype is associated with HOXB13 overexpression as shown *in vitro* and *in vivo*.

3.3 – HOXB13 promotes stromal recruitment via IL6 upregulation

Upon examining the histology of the MCF7 xenografts, we observed a more prominent stromal component (Figure 3.3a) in MCF7-HOXB13-overexpressing tumors compared to vector control, which showed growth as tight, uniformly spaced, carcinoma cells with little stromal presence. Because stromal recruitment is known to participate in breast cancer aggression (18, 53, 54), we hypothesized that mechanisms that drive HOXB13-overexpressing tumor growth may also contribute to tumor aggression through

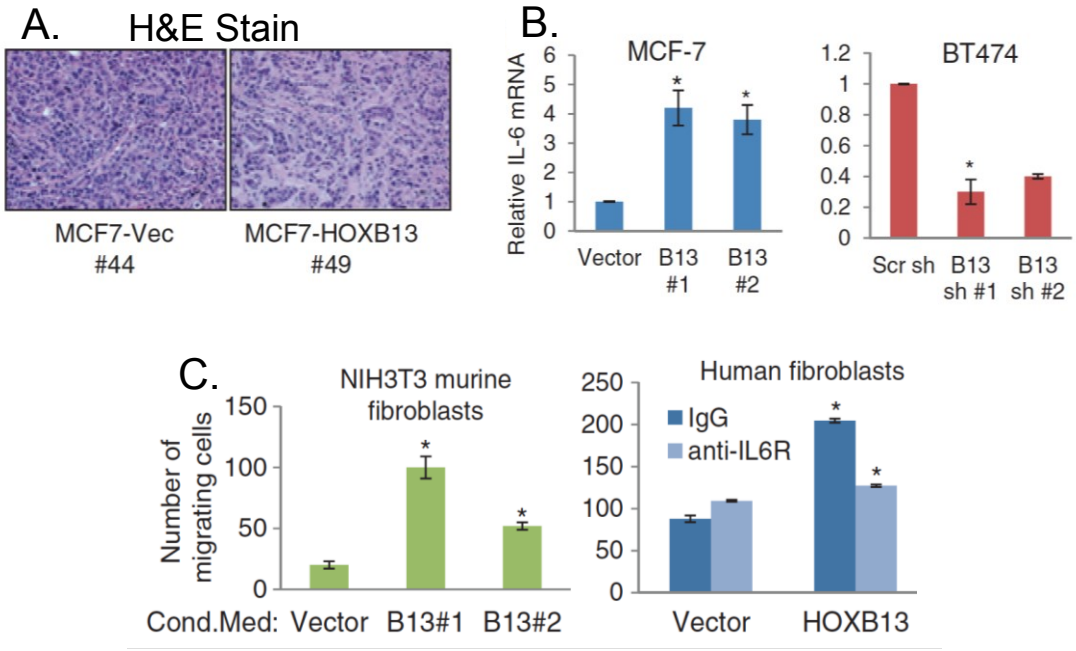

Figure 3.3. HOXB13 promotes stromal recruitment via IL6 upregulation. A, Hematoxylin and eosin-stained sections of MCF7 xenografts. MCF7 vector tumors on the left grow as uniform sheets of cancer cells, with little stromal component. In contrast, MCF7 HOXB13 tumors on the right have significantly more stromal infiltration. B, qPCR analysis of IL6 expression in MCF7 HOXB13 on the left and BT474 HOXB13-KD on the right compared with the control cells. C, Cell invasion/chemotaxis analysis using MCF7 HOXB13-conditioned media as the chemoattractant for untreated NIH3T3 fibroblasts or primary human fibroblasts pre-treated with IgG or anti-IL-6R antibody (*, P<0.01).

stroma-tumor crosstalk. Thus, we tested a panel of cytokines involved in stromal recruitment and cell invasion (not shown) by qPCR to test if their mRNA expression correlated with that of HOXB13. We found that interleukin-6 (IL6) is consistently expressed in high HOXB13-expressing MCF-7 but not in the MCF-7 vector transfected cell lines (Figure 3.3b).

In order to evaluate the ability of HOXB13-expressing breast cancer cells to recruit fibroblasts and to define the role of secreted-IL6 as a cytokine in this function, we performed fibroblast migration assays with both NIH3T3 murine fibroblasts and human fibroblasts. In a Boyden chamber assay, we tested fibroblast migration from inside the chamber to the outer membrane towards conditioned media collected from MCF-7 vector control or MCF7 HOXB13-overexpressing cell lines placed in the lower chamber (Figure 3.3c). We observed a significant increase in the number of migrating fibroblasts toward conditioned media from MCF7 HOXB13-overexpressing cells as opposed to the number migrating towards the medium from the MCF-7 vector control.

To determine if the migration is mediated largely through the presence of IL6 in the HOXB13-overxpressing tumor cell conditioned medium, we performed the following test. Upon pre-treatment of the human fibroblasts with a neutralizing anti-IL6R antibody, the increase in the number of migrating cells observed was abrogated, suggesting that by blocking the IL6 receptor on the migrating cells, their attraction towards the cytokine was significantly reduced. Thus, IL6 was the major stromal chemoattractant presented in the conditioned medium, which was secreted by the HOXB13 overexpressing carcinoma cells.

3.4 – IL6 is a direct transcriptional target of HOXB13

Because HOXB13 functions a transcription factor, we wanted to investigate the manner by which HOXB13-overexpression would result in the upregulation of IL6. This

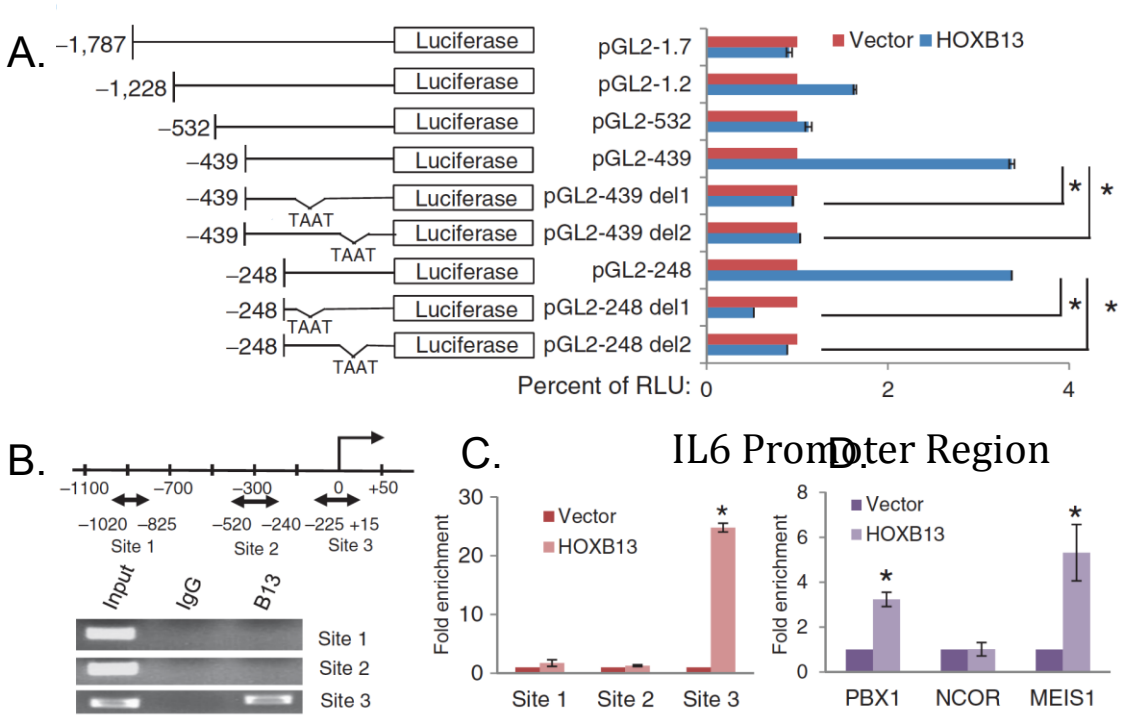

Figure 3.4. IL6 is a direct transcriptional target of HOXB13. A, IL6 promoter-luciferase construct maps on the left and luciferase activity of each construct, co-transfected with vector in red or pHOXB13 in blue, identifying a region 248 bp upstream of the TSS, including 2 HOX binding sequences, as critical to HOXB13-directed enhancement (*, P<0.01). B, Diagram of regions in the IL6 promoter with HOXB13 binding sites (double arrow). ChIP on MCF7 HOXB13 cells with either anti-HOXB13 antibody or control IgG shows Site 3 is enriched with HOXB13 expression (bottom). C, qPCR analysis of the 3 putative HOXB13 binding sites in the IL6 promoter after HOXB13 ChIP assay. D, qPCR analysis of HOXB13 binding Site 3 after PBX1, NCOR, or MEIS1 ChIP assay (*, P<0.01).

A.

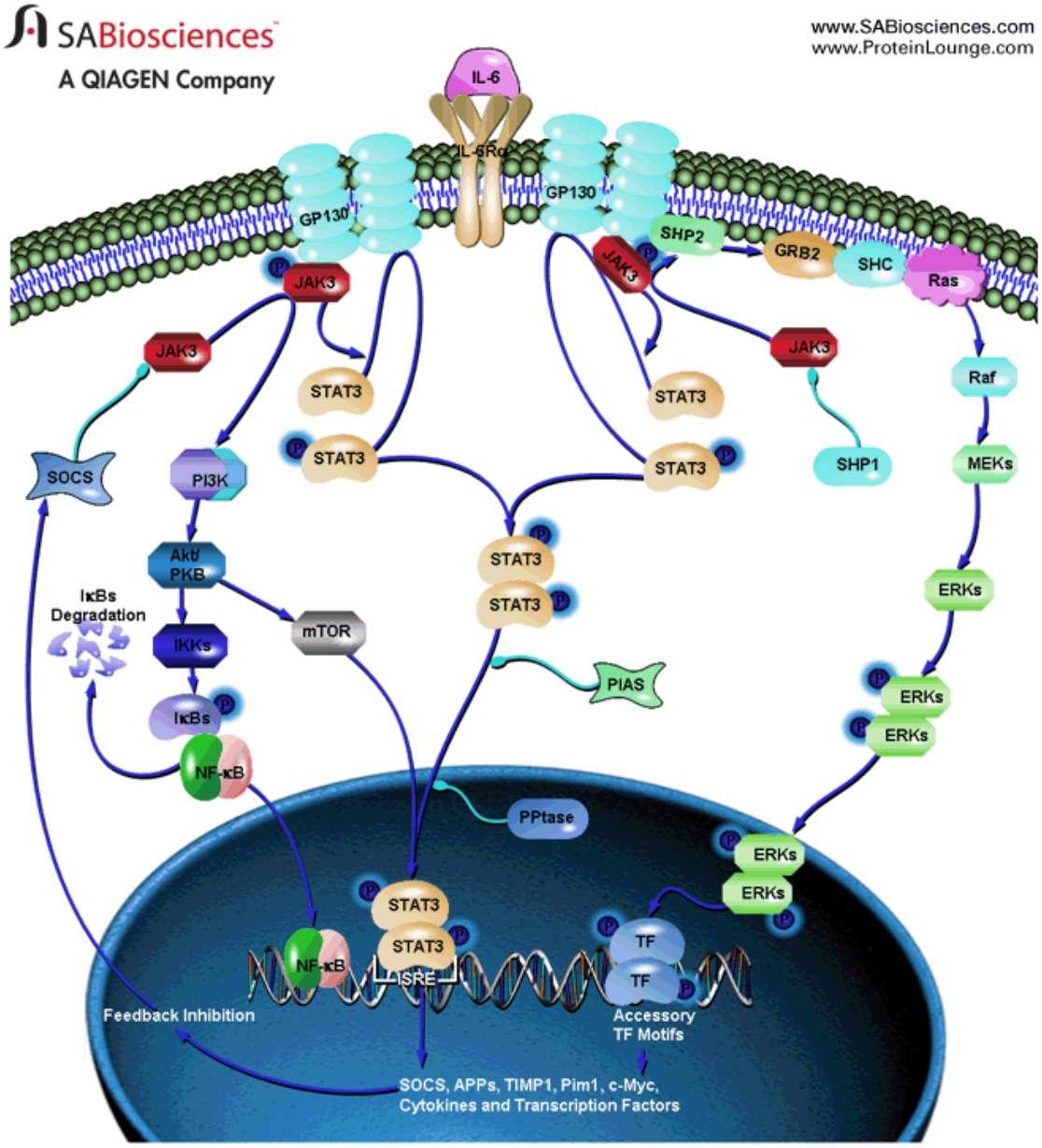

Figure 3.5a. IL6 signaling pathway (Accessed on August 6, 2014 from http://www.sabiosciences.com/pathway.php?sn=IL-6_Pathway.).

could occur via direct binding of HOXB13 to the IL6 promoter, and/or protein-protein interaction and binding of HOX co-factors, and the resulting activation of IL6 transcription. To evaluate if HOXB13, as a transcription factor, was directly driving IL6 transcription, we performed luciferase activity assays. Constructs containing 1.7 kb of the IL6 promoter and its deletions were linked to the luciferase reporter gene, and were transiently transfected into MCF7 cells along with HOXB13 expression plasmids. No significant activation of the luciferase gene was observed with the -1787 or -1228 bp or the -532 bp promoter fragments. However, four-fold activation was observed with the -439 bp construct, suggesting that negative transcriptional elements may reside in the longer constructs (Figure 3.4a). Deletion of the promoter sequence up to -248 retained the ability of HOXB13 to activate the luciferase construct. To test whether this activation is HOXB13-mediated, we prepared constructs that deleted each of the putative binding sites for HOXB13. Deletion of any one of three putative HOXB13 binding sites in the -248 bp construct within the IL6 promoter abrogated luciferase activity, indicating the requirement for all three binding sites for HOXB13 mediated promoter activation as tested by this assay.

In order to better define which putative site in IL6 is the vital binding site for HOXB13, we interrogated the three putative HOX binding sites identified above in the -248 bp fragment of the IL6 promoter region close to the transcriptional start site by performing a ChIP assay. Control IgG or anti-HOXB13 antibody was used to immunoprecipitate HOXB13 complexes crosslinked to the IL6 promoter DNA fragments in MCF7 HOXB13-overexpressing cells. We observed that site 3 (-225 to +15 bp of the TSS), but not the other two sites, was enriched for HOXB13 binding in MCF7 HOXB13-overexpressing cells as shown by PCR products fractionated on an agarose gel (Figure 3.4b). qPCR analysis of the three putative IL6-promoter binding sites also showed enrichment of site 3 specifically in

MCF7 HOXB13-overexpressing cells compared to vector control (Figure 3.4c). To investigate possible binding of HOXB13 to HOX co-factors vital for transcriptional regulation, we performed ChIP assays on the three different HOX co-factors PBX1, NCOR, and MEIS1, and observed enrichment at site 3 for the co-activators PBX1 and MEIS1, but not of the co-repressor NCOR (Figure 3.4d). These findings support the hypothesis that HOXB13 binds the IL6 promoter directly and promotes IL6 transcription.

3.5 – The IL6 pathway is activated in HOXB13-expressing cells

IL-6 is known to promote tumorigenesis through multiple mechanisms, such as cell proliferation and stromal recruitment (55-57) and causes these effects via phosphorylation of the downstream effectors STAT3, PI3K/AKT and mTOR (Figure 3.5a – IL6 pathway diagram) of its pathway. Thus, we decided to interrogate the activation of the IL6 pathway in our HOXB13-expressing cell lines by western blot analysis. Consistent with signaling activation as a result of IL6 ligand action on IL6R, we observed that although total-STAT3 levels did not change, there was increased phosphorylated STAT3 (P-STAT3) at tyrosine 705 in MCF7 HOXB13-overexpressing cells compared to vector control. Similar relationships were also observed between AKT at serine 473 and in P70S6K and 4EBP1, the downstream effectors of mTOR action in the MCF7 HOXB13-overexpressing lines. BT474-Scr cells exhibited higher phosphorylated STAT3, AKT, P70S6K, and 4EBP1, where these levels were reduced in the BT474 HOXB13-KD clones, without any significant changes in the levels of the total proteins, confirming specific induction of IL6 pathway components in our HOXB13-expressing cell lines (Figure 3.5b).

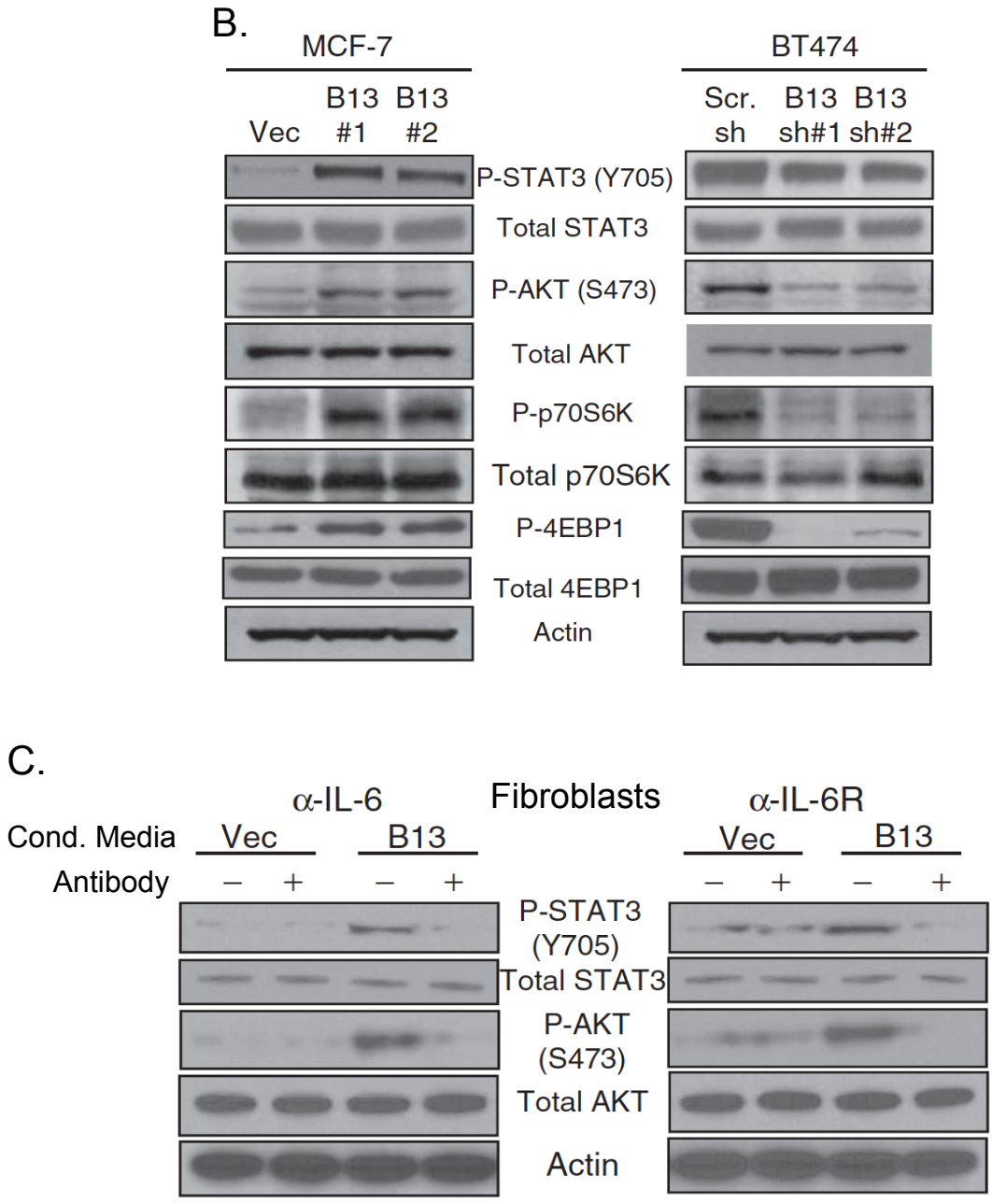

Figure 3.5b and 3.5c. IL6 signaling pathway activation in HOXB13-expressing breast cancer cells and recruited fibroblasts. A, Western blot analysis of p-STAT3 (Y705), p-AKT (S473), p-p70S6K, and p-4EBP1 in MCF7 HOXB13 and BT474 HOXB13-KD cells compared with control cells. B, Western blot analysis of p-STAT3 (Y705) and p-AKT (S473) in human fibroblasts after treatment with conditioned media from MCF7 vector or MCF7 HOXB13 cells, with and without treatment of either anti-IL6 on the left or anti-IL6R antibody on the right.

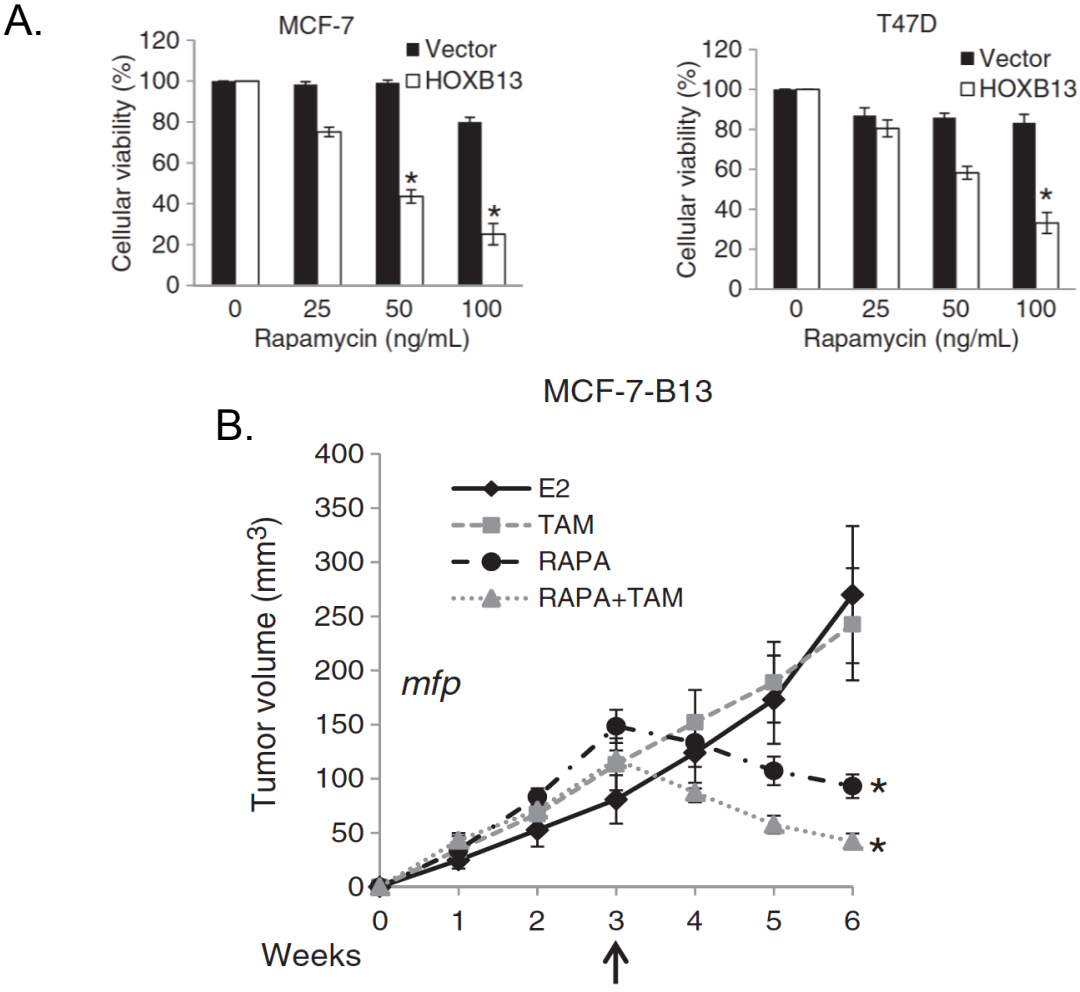

Figure 3.6. Rapamycin can inhibit proliferation of HOXB13-expressing breast cancer cells *in vitro* and *in vivo*. A, Cell viability analysis following treatment with 25, 50, and 100 ng/mL rapamycin in MCF7 HOXB13 and T47D HOXB13 cells compared with vector control cells (*, P<0.005). B, Growth of MCF7 HOXB13 xenografts implanted in athymic mouse mammary fat pad (mfp). Xenografts grew in the presence of estrogen (E2) for 3 weeks; mice were then treated with TAM (square), rapamycin (circle), or rapamycin and TAM (triangle) for 3 weeks. Tumor volume was significantly smaller in rapamycin alone and rapamycin and TAM treated groups compared with either vehicle or tamoxifen alone (*, P<0.005).

3.6 – Rapamycin can inhibit proliferation of HOXB13-expressing breast cancer cells *in vitro* and *in vivo*

After verifying activation of the IL6 pathway in our HOXB13-expressing cells, we looked to interrogate the downstream effector mTOR as a targetable aspect of the IL6 pathway by inhibiting mTOR activity in our cells with rapamycin. Upon testing the cell viability of MCF7 and T47D HOXB13-overexpressing cell lines upon rapamycin treatment, we observed that our HOXB13-overexpressing cells were specifically susceptible to rapamycin when compared to vector control (Figure 3.6a). Given the suppressive effect of rapamycin on HOXB13-overexpressing cells *in vitro*, we further tested sensitivity of our HOXB13-overexpressing cells to rapamycin *in vivo*, also evaluating if there would be an additive or synergistic effect of using rapamycin with TAM. After MCF7 HOXB13-overexpresing xenografts were grown for 3 weeks, the mice were treated with estrogen alone, TAM alone, rapamycin alone, or rapamycin and tamoxifen in combination. Rapamycin alone and in combination with TAM caused marked tumor regression (with no additive nor synergistic effect for rapamycin treatment in combination with TAM) and was significantly more effective than TAM alone (Figure 3.6b), showing that by targeting the activated mTOR pathway in HOXB13-overexpressing cells, we could specifically inhibit their proliferation. The observations in this paper regarding HOX13 expression show that HOXB13 is overexpressed in ER-positive breast cancer and metastases. The experiments conducted in cell culture and *in vivo* suggest that HOXB13 overexpression promotes TAMR as well as stromal recruitment through IL6 upregulation mediated by the direct binding of HOXB13 to the IL6 promoter, resulting in an induction of IL6 transcription. We also found that in the HOXB13-expressing cells themselves, the IL6 pathway is upregulated and that by targeting

the downstream effector mTOR with rapamycin, we can inhibit HOXB13-overexpressing TAMR cell growth *in vitro* and *in vivo*.

CHAPTER 4

Discovery of novel targets associated with HOXB13-expression

4.1 – Novel targets display correlation between mRNA expression and HOXB13-expression

We used a two-pronged approach to our goal of discovering genes that are altered by HOXB13 expression. The first, using microarray analysis to discover new targets associated with HOXB13-expression in our cell line models, and the second, to better define other downstream effectors of the IL-6 pathway that may be more accessible, more effective targets to inhibit HOXB13-mediated-TAMR. In order to investigate genes possibly regulated by HOXB13-overexpression that may contribute to TAMR, we performed an Affymetrix GeneChip PrimeView Human Gene Expression Array on our BT474 cell line panel, which consisted of BT474-Scramble (BT474-Scr) control, which exhibits endogenous high expression of HOXB13 as well as is inherent tamoxifen resistant, and BT474 HOXB13 knockdown (KD) clonal cell lines that we ourselves created (Figure 4.1). The heat map generated through microarray analysis shows differential expression between the BT474 scramble control and HOXB13 knockdown cell lines (Figure 4.2).

From this analysis we selected the top 5 upregulated and the bottom 5 downregulated genes from our list of differentially expressed genes (Table 4.1) and validated their expression in the BT474 cell line panel by RT-qPCR (Figure 4.3), and observed that upregulated genes in the microarray were overexpressed in scramble compared to the knockdown-HOXB13 lines and vice-versa with the downregulated genes. From the list of differentially expressed genes, we focused on two possible target candidates, DKK1 and MERTK.

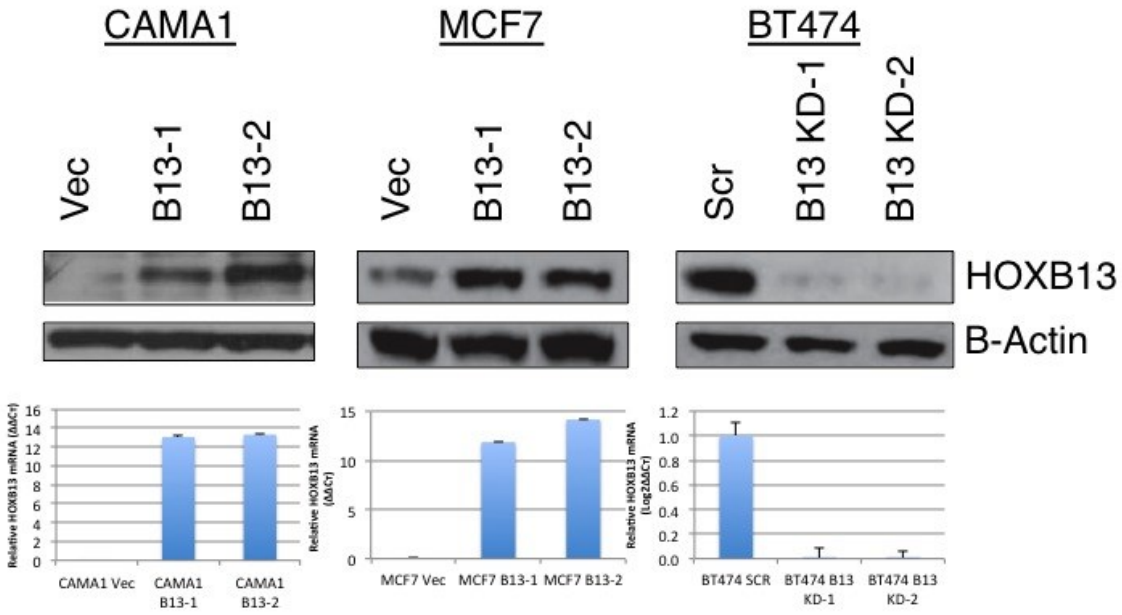

Figure 4.1. Validation of HOXB13-expressing cell line model panels. Western blot and qPCR analysis of HOXB13 expression in CAMA1 and MCF7 HOXB13-overexpressing cell line panels compared with vector controls confirm HOXB13-overexpression for both mRNA and protein levels; reciprocally, the BT474 HOXB13-KD cell line panel also confirms effective HOXB13 knockdown.

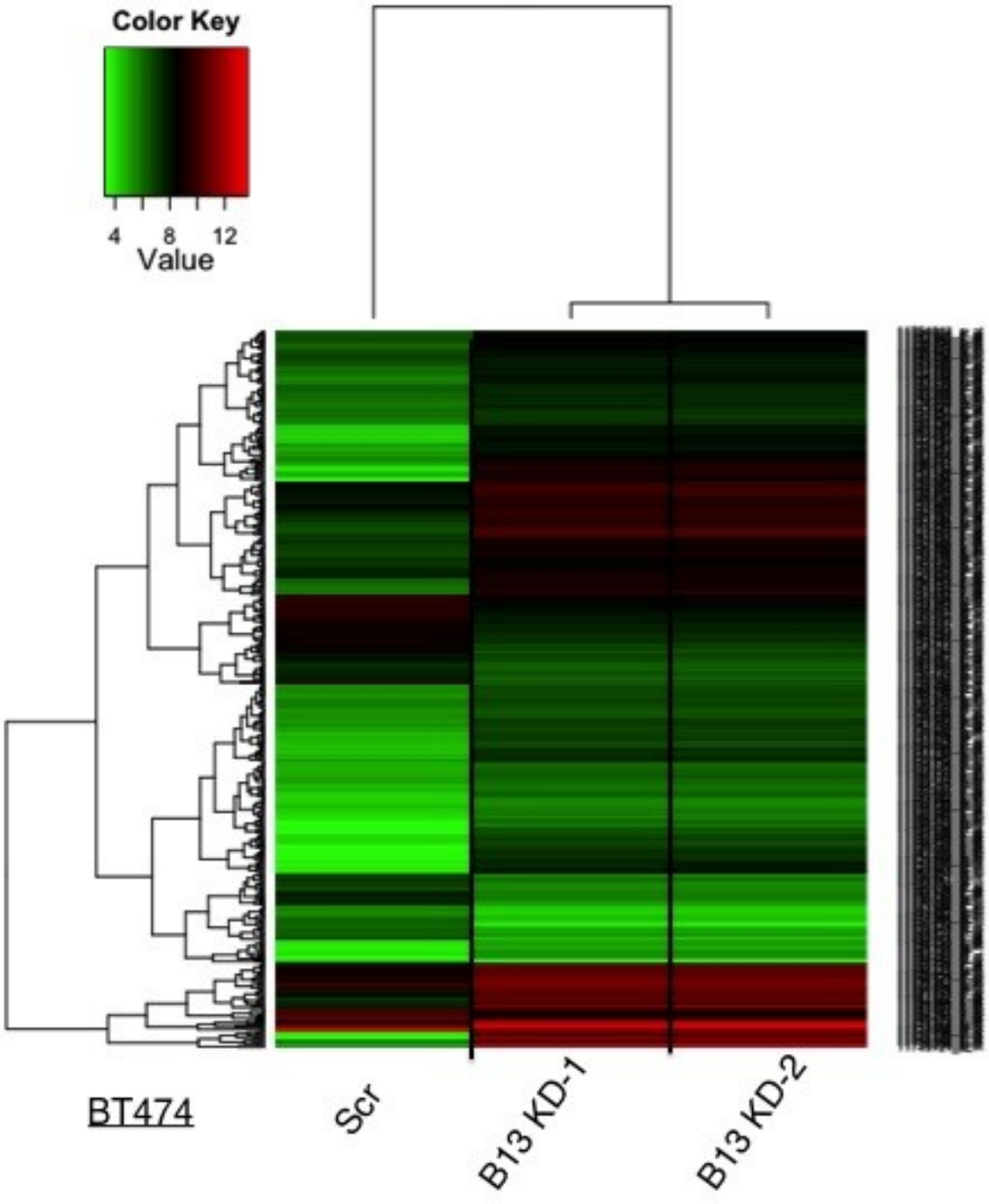

Figure 4.2. Differential expression observed after microarray analysis between BT474 scramble control and the BT474 HOXB13-KD cell lines. Unsupervised hierarchical clustering resulted in the generation of two separate clusters between the BT474 scramble control and the BT474 HOXB13-KD cell lines.

Gene	Log2FC	Function
CYP2A6	2.97	Key enzyme for metabolism of xenobbiotics; E2-induced; cytochrome P450 family
BASP1	2.89	Brain acid soluble protein 1
CST1	2.23	Cystatin-SN
DKK1	2.07	Dickkopf-related protein 1, inhibitor of Wnt signaling, overxps in breast cancer
COLEC12	2.06	Collectin sub-family member 12
MERTK	1.14	RTK, promotes invasion in glioblastoma
TYRP1	-5.34	Overexpression assoc. with normal tissue or non-metatstatic tissue
XAGE1A	-5.69	XAGE subfamily of GAGE proteins
LGALS1	-6.45	Galectin-1, implicated in modulating cell-cell and cell-matrix interactions
MAGEA3	-7.78	Melanoma-associated antigen 3, tumor-specific antigen of melanoma and lung cancer
SERPINA6	-8.25	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6, major transport protein for glucorticoids and progestins

Table 4.1. Top 5 upregulated and bottom 5 downregulated genes based on BT474 HOXB13-KD cell line panel microarray analysis. Log2FC = Log2 Fold Change.

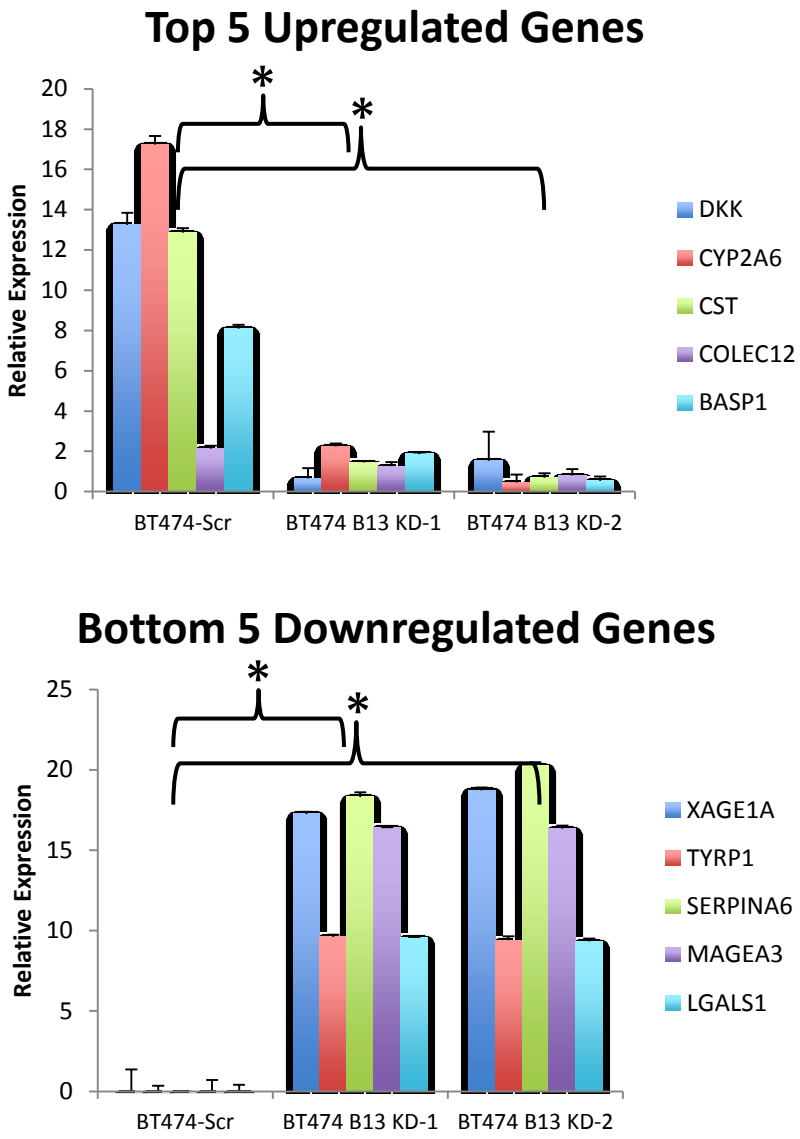

Figure 4.3. Validation of target gene mRNA expression correlation with HOXB13-expression. qRT-PCR analysis of the top 5 upregulated and bottom 5 putative target genes' mRNA expression in the BT474 HOXB13-KD cell line panel. (*, P<0.01)

4.2 – Dickkopf-related protein 1 (DKK1)

Dickkopf-related protein 1 or DKK1 is a key inhibitor of the canonical Wnt/Beta-catenin (B-cat) signaling pathway and is a direct downstream target of B-cat/TCF/LEF transcriptional activation (58). Because DKK1 overexpression has been observed in a wide variety of cancers including breast (59), and because B-cat expression is associated with poor prognosis in breast cancer patients (60), this suggested an unknown mechanism for an oncogenic role for DKK1.

We hypothesized that if DKK1 overexpression in HOXB13-overexpressing TAMR breast cancer correlated with an upregulation of Wnt signaling, we would observe an increase in activated (free) B-cat, binding of activated B-cat to the TCF/LEF cofactor complex, and induced upregulation of Wnt target genes. Therefore, we assessed mRNA expression of Wnt ligand and co-receptors, as well as the expression of Wnt target genes in our HOXB13-cell lines through qPCR.

Assessment of DKK1 mRNA expression in our HOXB13-overexpressing cell line panels found that its overexpression correlated with HOXB13 overexpression in CAMA1 HOXB13 overexpressing clones and its downregulation correlated with HOXB13 knockdown in BT474 cells (Figure 4.4). HOXB13-overexpression in MCF7 did not correlate with DKK1 overexpression.

Both Wnt ligand Wnt3a and Wnt co-receptor FZD1 mRNA expression was found to be downregulated in our BT474 B13-knockdown cell lines and overexpressed in only one of our CAMA1 HOXB13-overexpressing clones (Figure 4.5), which would suggest upregulation of the Wnt pathway. In spite of this result, we observed the upregulation of only one canonical Wnt target gene, Axin2 (Figure 4.6). Western blot analysis of activated B-cat protein in these cell lines showed that in all cell lines of the CAMA1 HOXB13-

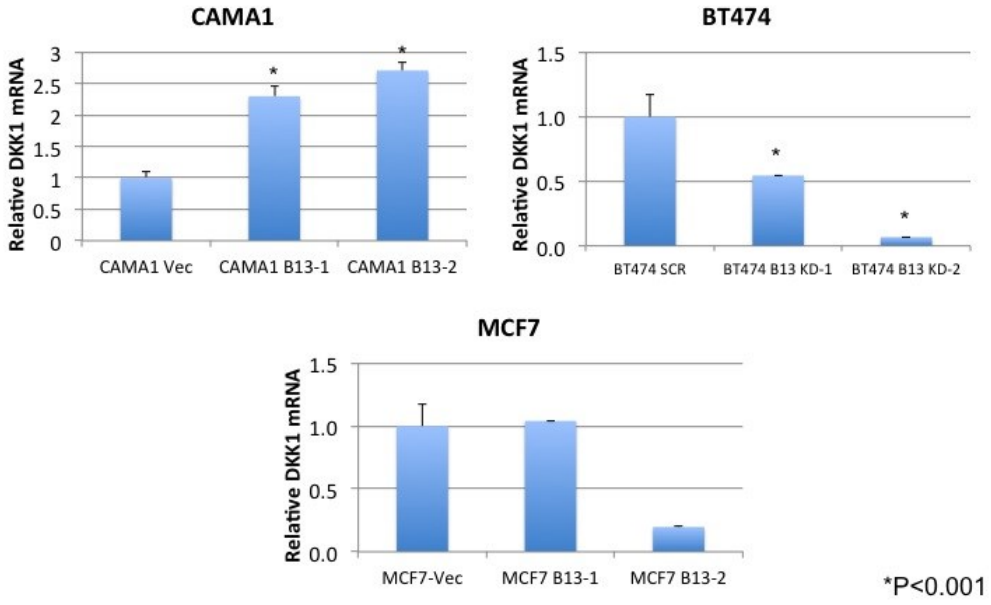

Figure 4.4. DKK1 overexpression correlates with HOXB13-expression in CAMA1 and BT474 models. qRT-PCR analysis of DKK1 mRNA levels in MCF7 and CAMA1 HOXB13-overexpressing cells, as well as BT474 HOXB13-KD cells compared with control cells. (*, P<0.001).

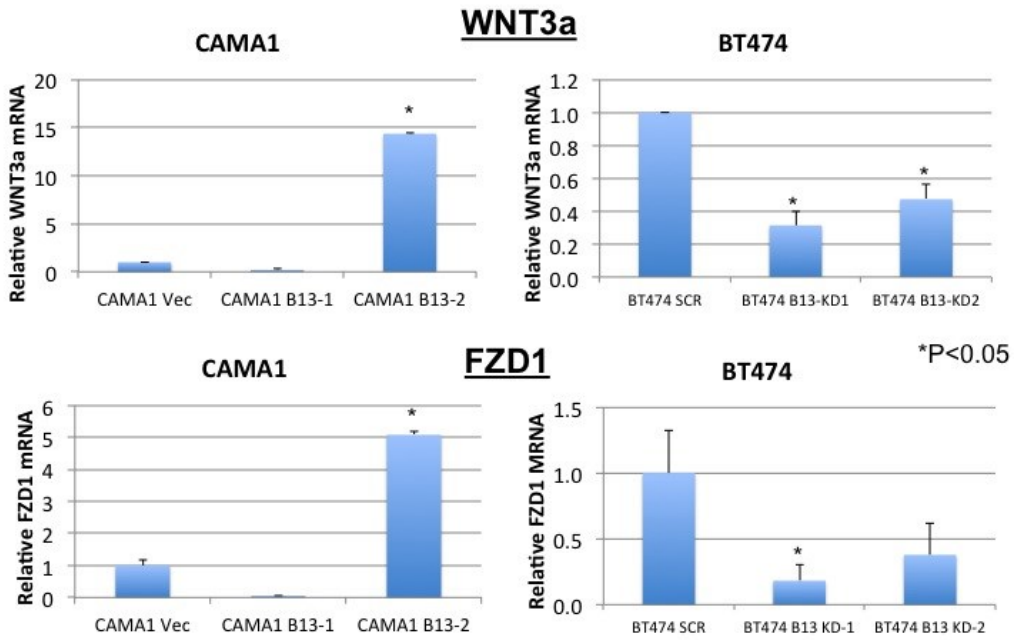

Figure 4.5. Wnt ligand and co-receptor overexpression correlates with HOXB13-expression. qRT-PCR analysis of WNT3a and FZD1 mRNA levels in CAMA1 HOXB13-overexpressing cells and BT474 HOXB13-KD cells compared with control cells. (*, P<0.05).

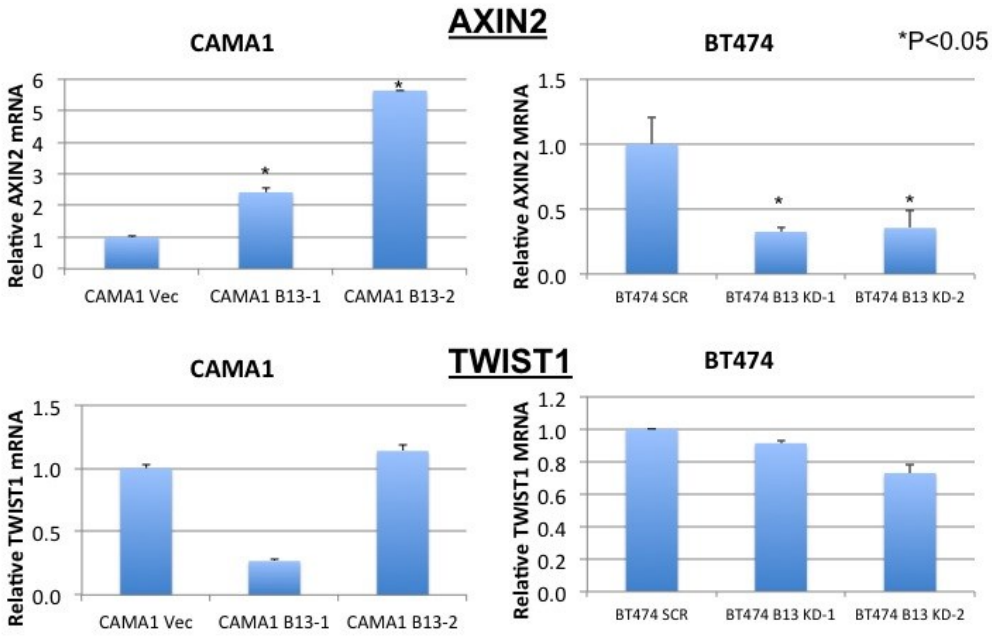

Figure 4.6. Overexpression of Wnt target gene AXIN2 correlates with HOXB13-expression; expression of Wnt target gene TWIST1 does not. qRT-PCR analysis of AXIN2 and TWIST mRNA levels in CAMA1 HOXB13-overexpressing cells and BT474 HOXB13-KD cells compared with control cells (*, P<0.05).

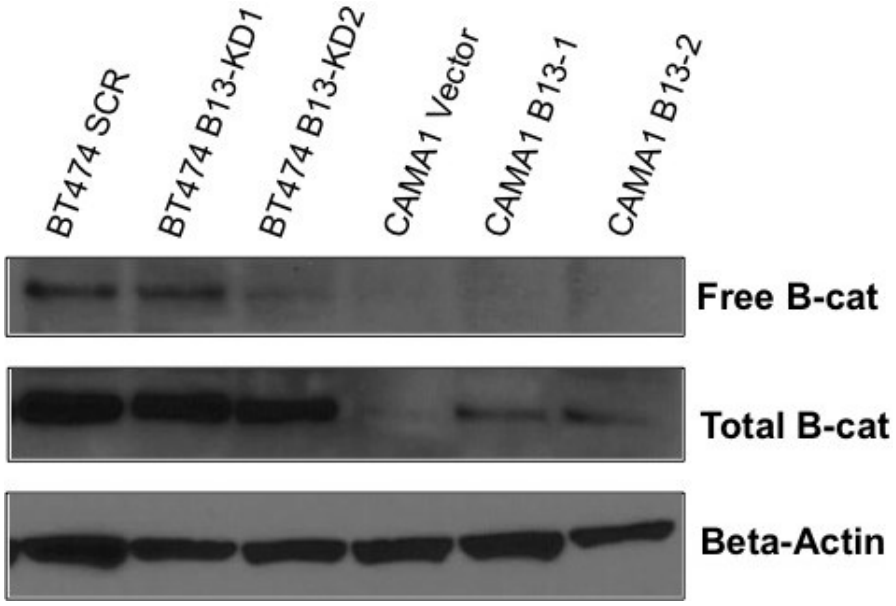

Figure 4.7. HOXB13 expression does not induce WNT signaling. Western blot analysis of free (activated) B-cat in BT474 HOXB13-KD cells and CAMA1 HOXB13-overexpressing cells compared with control cells.

overexpressing panel, there is little to no activated B-cat. In the BT474 HOXB13-KD panel, we observed levels of activated B-cat in the scrambled DKK1/HOXB13 overexpressing cell line, however, only in Knockdown 2 was a decrease in activated B-cat observed (Figure 4.7).

These results suggested that upregulation of DKK1 in HOXB13-overexpressing cells seems to have no effect on canonical Wnt signaling/B-cat activation. Because DKK1 is a direct Wnt target gene, increased Wnt signaling would induce its transcription; deregulation of Wnt signaling may be so strong that it may override DKK1 inhibition, therefore, making DKK1 overexpression an artifact of aberrant Wnt signaling in HOXB13-mediated TAMR breast cancer.

4.3 – C-mer proto-oncogene tyrosine kinase (MERTK)

MERTK is also known as the C-mer proto-oncogene tyrosine kinase and is a member of the TYRO3/AXL/MER receptor kinase family. It is a potent mediator of pro-survival and anti-apoptotic signaling pathways and is an upstream activator of MAPK, PI3K/Akt, and Jak/Stat pathways. Downstream of MERTK, there are additional signaling pathways important to cytokine production and enhanced migration/invasion (61). We observed MERTK overexpression in correlation with HOXB13 overexpression in our CAMA1 and BT474 cell lines; however, this was not observed in MCF7 (Figure 4.8).

Studies have shown the microRNA miR-126 to be commonly silenced in breast cancer and shown to suppress metastasis through inhibition of its target genes, among which is MERTK (62). This study, highlighting the seemingly important role of MERTK in breast cancer metastasis, prompted us to investigate MERTK mRNA expression in our store of patient tumor samples; however, in spite of expecting an increase in MERTK expression in a panel of patient tumor samples. However, contrary to the predicted increase in MERTK

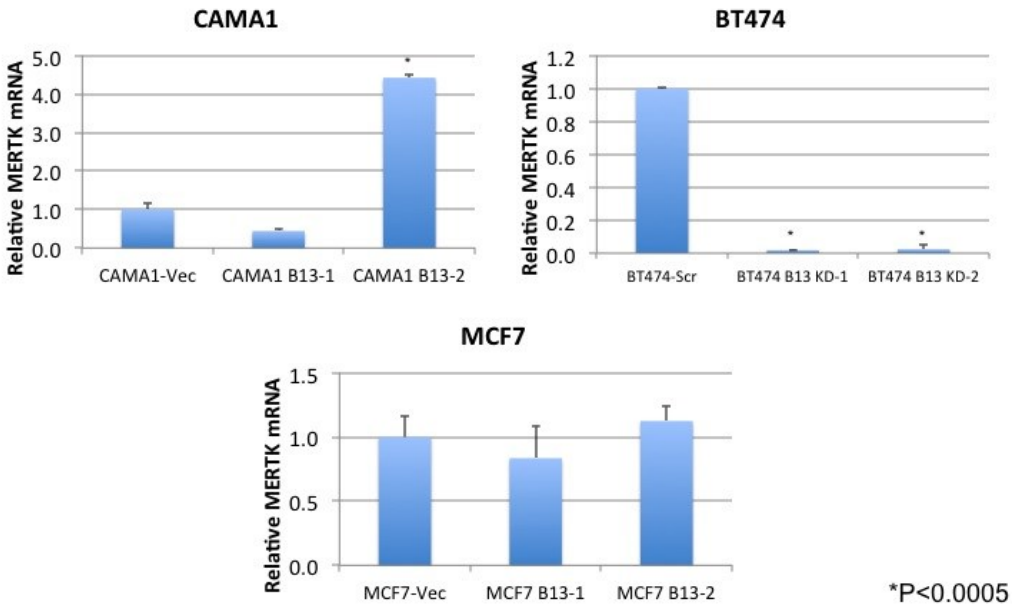

Figure 4.8. MERTK overexpression correlates with HOXB13-expression in CAMA1 and BT474 models. qRT-PCR analysis of MERTK mRNA levels in MCF7 and CAMA1 HOXB13-overexpressing cells, as well as BT474 HOXB13-KD cells compared with control cells. (*, P<0.0005).

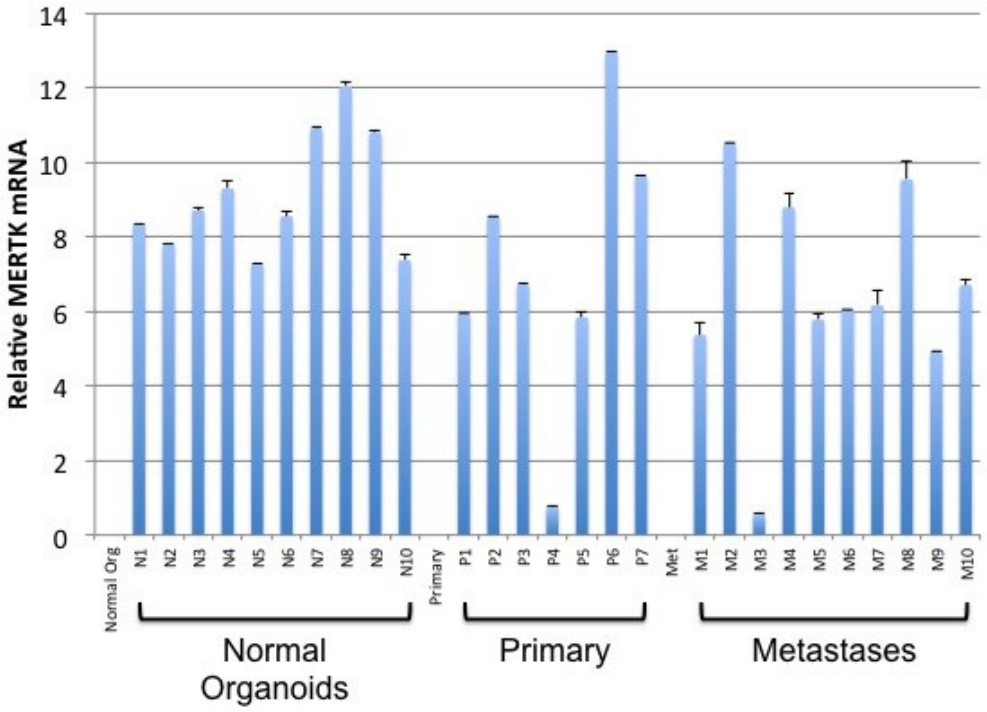

Figure 4.9. No trend of MERTK overexpression observed in primary breast cancers and distant metastases. qRT-PCR analysis of MERTK expression in normal breast organoids, ER-positive primary breast tumors, and metastases. (NS).

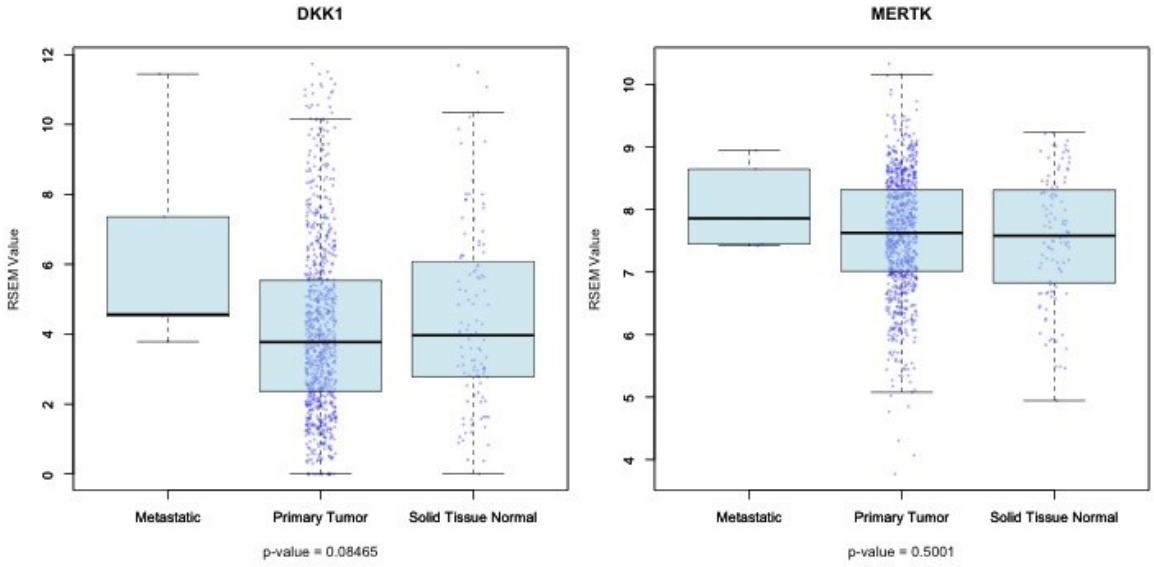

Figure 4.10. Diminutive change in DKK1 and MERTK mRNA expression between normal, tumor, and metastatic TCGA samples. (NS).

expression in tumor samples, there was no difference in MERTK levels between normal organoids, and both primary and metastatic cancer samples (Figure 4.9). It is likely that activated MERTK forms may be overexpressed in breast cancer; thus, this hypothesis remains to be tested.

4.4 – The Cancer Genome Atlas (TCGA) database analysis for DKK1 and MERTK

As a final effort to further probe these potential targets regarding tumor aggression and metastasis, we queried the TCGA database for both DKK1 and MERTK, where we did not see much change in expression between normal and tumor; also, the sample size was too small to feel confident about its overexpression in metastatic samples (Figure 4.10).

Conclusions

Based on our mixed observations regarding the role of DKK1 and MERTK in HOXB13-mediated TAMR, it is obvious that more detailed analysis is needed to determine the contributions of these genes to breast cancer.

CHAPTER 5

In vitro targeting of the IL-6 pathway in HOXB13-expressing breast cancer

5.1 – Fibroblast migration toward HOXB13-expressing cell line conditioned media is abrogated upon anti-IL-6R antibody Tocilizumab (TOC) treatment

In addition to the mTOR component of the IL6 pathway that was the focus of our investigation (33), we next investigated other important components of the pathway such as the IL6 receptor itself, STAT3, and PI3K/Akt, upstream of mTOR, by interrogating the effects of various inhibitors (Table 5.1) against these components on our HOXB13-expressing cell line panels.

Tocilizumab (TOC) is a humanized monoclonal anti-IL-6R antibody, kindly donated for our studies by Genentech, that is FDA approved and is currently used to treat rheumatoid arthritis (63). After treatment of MCF7 HOXB13-overexpressing and BT474 HOXB13-KD cells with increasing doses of TOC, we observed a slight decrease in MCF7 HOXB13-overexpressing cell viability compared to vector control. However, we did not see much change, if at all, in the cell viability of BT474-Scr cells compared to BT474 HOXB13-KD clones upon TOC treatment (Figure 5.1). This lack of cytotoxic effects of TOC on the tumor cells could likely be attributed to their effect on the stroma rather than the tumor cells themselves. Indeed, the first hint of collaboration of the fibroblasts in HOXB13-directed tumorigenesis was obtained from our observation that HOXB13 overexpressing tumors had a marked stromal component and a desmoplastic response typical of aggressive tumors.

As a first step, we investigated the effect of TOC on fibroblast migration in Boyden chamber assays. With the exposure of human mammary fibroblast in Boyden chambers to conditioned media from either MCF7 HOXB13-overexpressing or MCF7 vector control

Drug	Target	HOXB13-Specific Cell Viability Inhibition
Tocilizumab	**IL6R**	**None***
Niclosamide	STAT3	None
AS-605240	PI3K	None
BEZ235	**PI3K**	**Strong**
GDC-0941	PI3K	None
GSK1059615	PI3K	Slight
IC-87114	PI3K	Slight
LY294002	**PI3K**	**Strong**
PI-103	**PI3K**	**Strong**
PIK-90	PI3K	Slight
TG100-115	PI3K	Slight
TGX-221	PI3K	None
XL147	PI3K	None
ZSTK474	PI3K	Slight

Table 5.1. Panel of IL6 pathway inhibitors tested against HOXB13-expressing cell line panels. Rows in bold indicate drugs further pursued in our studies; *TOC did not specifically inhibit HOXB13-specific cell viability but did inhibit HOXB13-specific fibroblast migration and therefore was pursued.

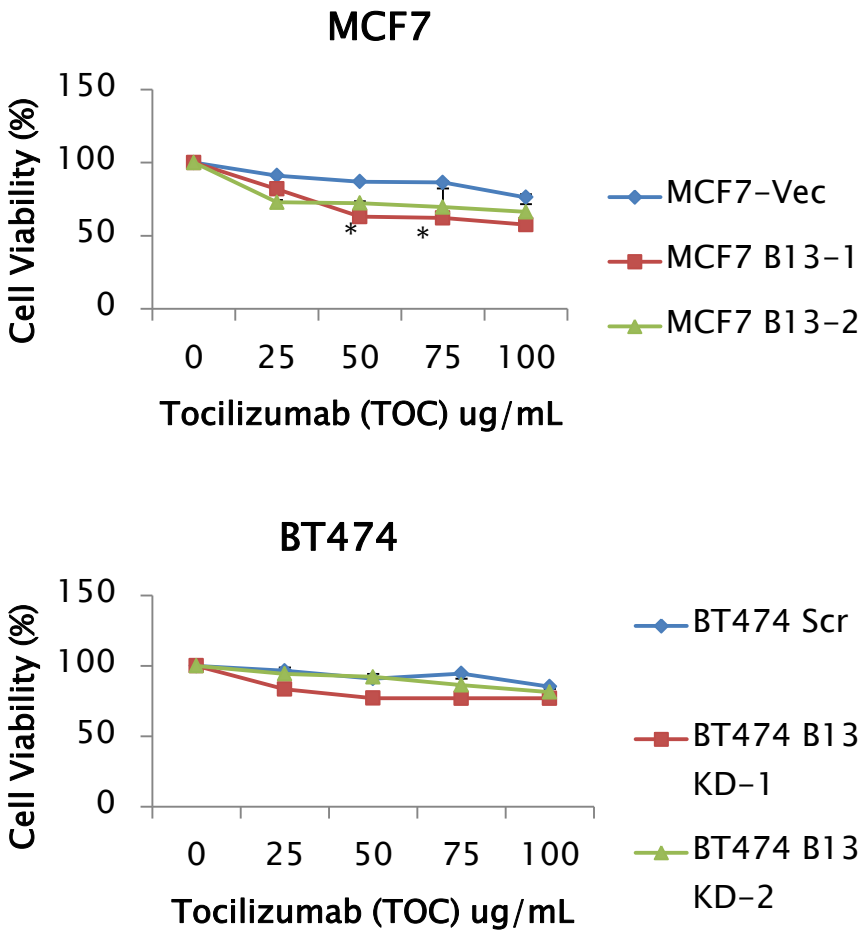

Figure 5.1. TOC does not significantly inhibit viability of HOXB13-overexpressing cells in culture. MTT cell viability after treatment with increasing doses of TOC in MCF7 HOXB13-overexpressing cells compared with MCF7 vector control and BT474-Scr cells compared with BT474 HOXB13-KD. (*, $P<0.05$ for both MCF7 B13-1 and MCF7 B13-2).

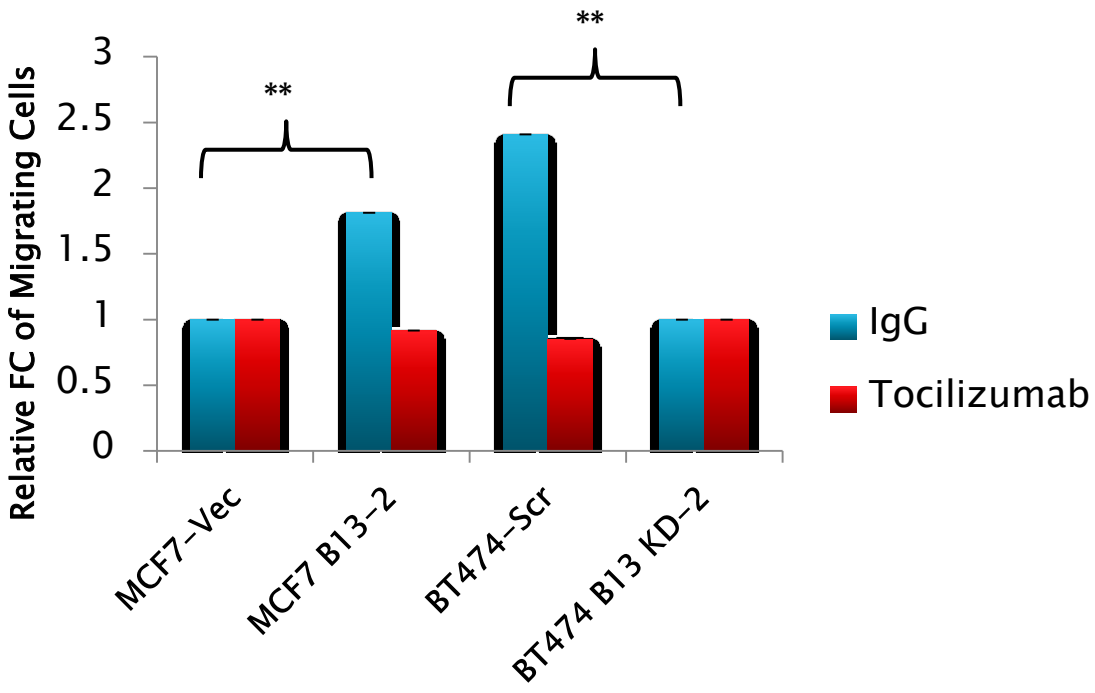

Figure 5.2. TOC inhibits fibroblast migration toward HOXB13-expressing conditioned media. Cell invasion/chemotaxis analysis using conditioned media from MCF7 vector control, MCF7 HOXB13-overexpressed, BT474-Scr, or BT474 HOXB13-KD cells as the chemoattractant for primary human fibroblasts untreated or pre-treated with IgG or TOC (**, P<0.001).

cells, BT474-Scr, or BT474 HOXB13-KD cells, we observed that, as in our previous studies that there was a marked increase in the number of migrating fibroblasts toward MCF7 HOXB13-overexpressing or BT474-Scr conditioned media as opposed to MCF7 vector control or BT474 HOXB13-KD conditioned media and that this increase in migration was abrogated upon TOC pre-treatment and thereby blocking of the IL6 receptors of the fibroblasts (Figure 5.2).

5.2 – Niclosamide, inhibitor of STAT3 activation, is ineffective at inhibiting cell viability of HOXB13-overexpressing cells

Downstream of IL6R signaling is the activation of STAT3, which was inhibited in HOXB13-expressing cells by treatment of the cells with Niclosamide. Both MCF7 HOXB13-overexpressing and BT474 HOXB13-KD cell models did not show decreased cell viability with increasing doses of Niclosamide (Figure 5.3).

5.3 – NVP-BEZ-235, PI3K inhibitor, is effective at inhibiting HOXB13-specific cell viability

We interrogated the PI3K/Akt pathway, by testing multiple PI3K inhibitors against our MCF7 HOXB13-overexpressing cell line panel. Out of twelve drugs tested: AS-605240, BEZ235, GDC-0941, GSK1059615, IC-87114, LY294002, PI-103, PIK-90, TG100-115, TGX-221, XL147, and ZSTK474, the three drugs presenting the strongest HOXB13-specific decrease in cell viability were further interrogated not only in the MCF7 HOXB13-overexpressing cell line panel, but also in our BT474 HOXB13-KD cell line panel. Increasing doses of NVP-BEZ235 (64) induced HOXB13-specific decrease in cell viability for both MCF7 HOXB13-overexpressing cell lines as well as in the BT474-Scr line compared to vector control and KD cell lines respectively, making BEZ a promising drug candidate (Figure 5.4). LY294002 showed HOXB13-specific cell death at lower doses for

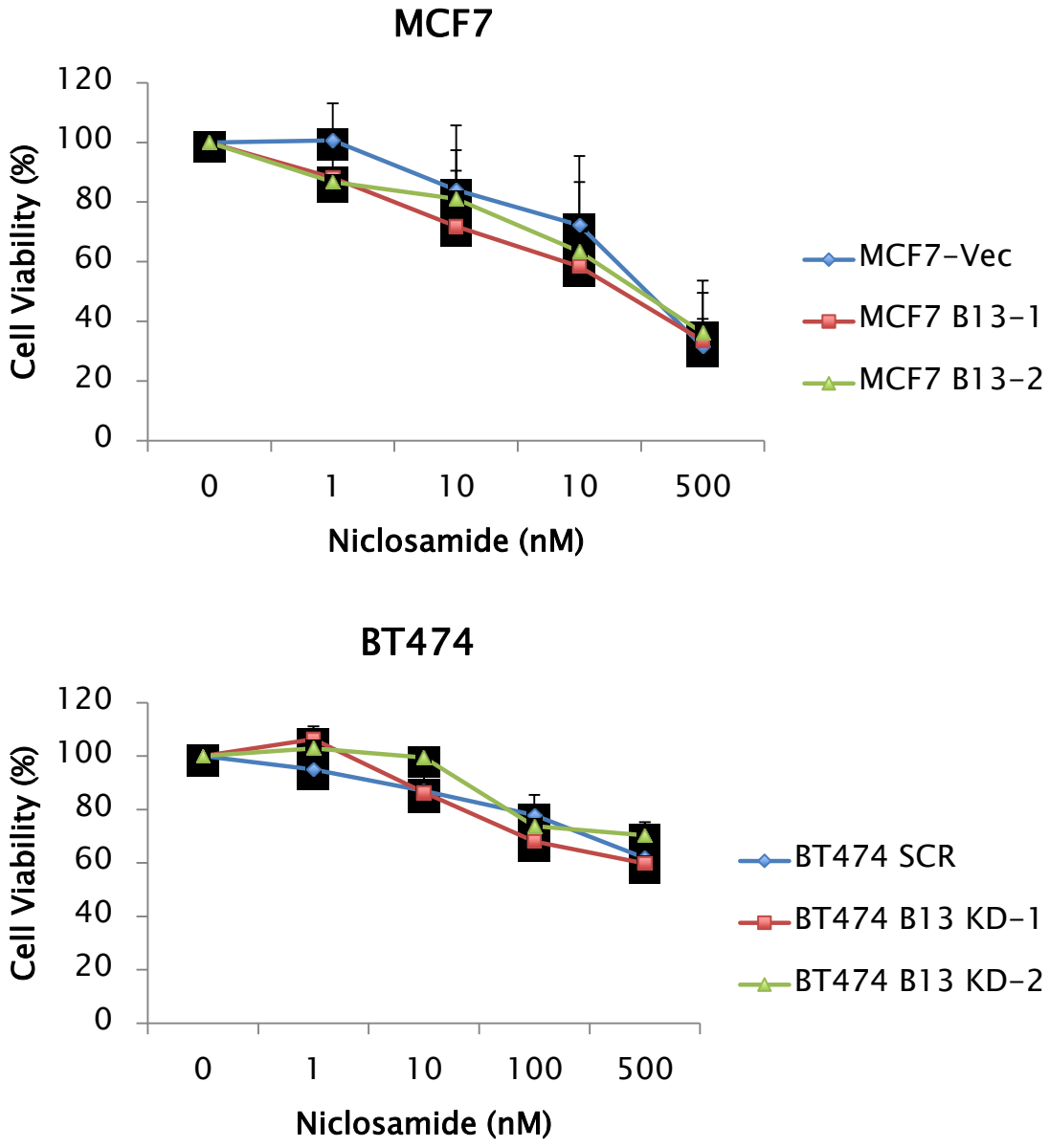

Figure 5.3. Niclosamide does not significantly inhibit viability of HOXB13-overexpressing cells in culture. MTT cell viability after treatment with increasing doses of niclosamide in MCF7 HOXB13-overexpressing cells compared with MCF7 vector control and BT474-Scr cells compared with BT474 HOXB13-KD. (NS)

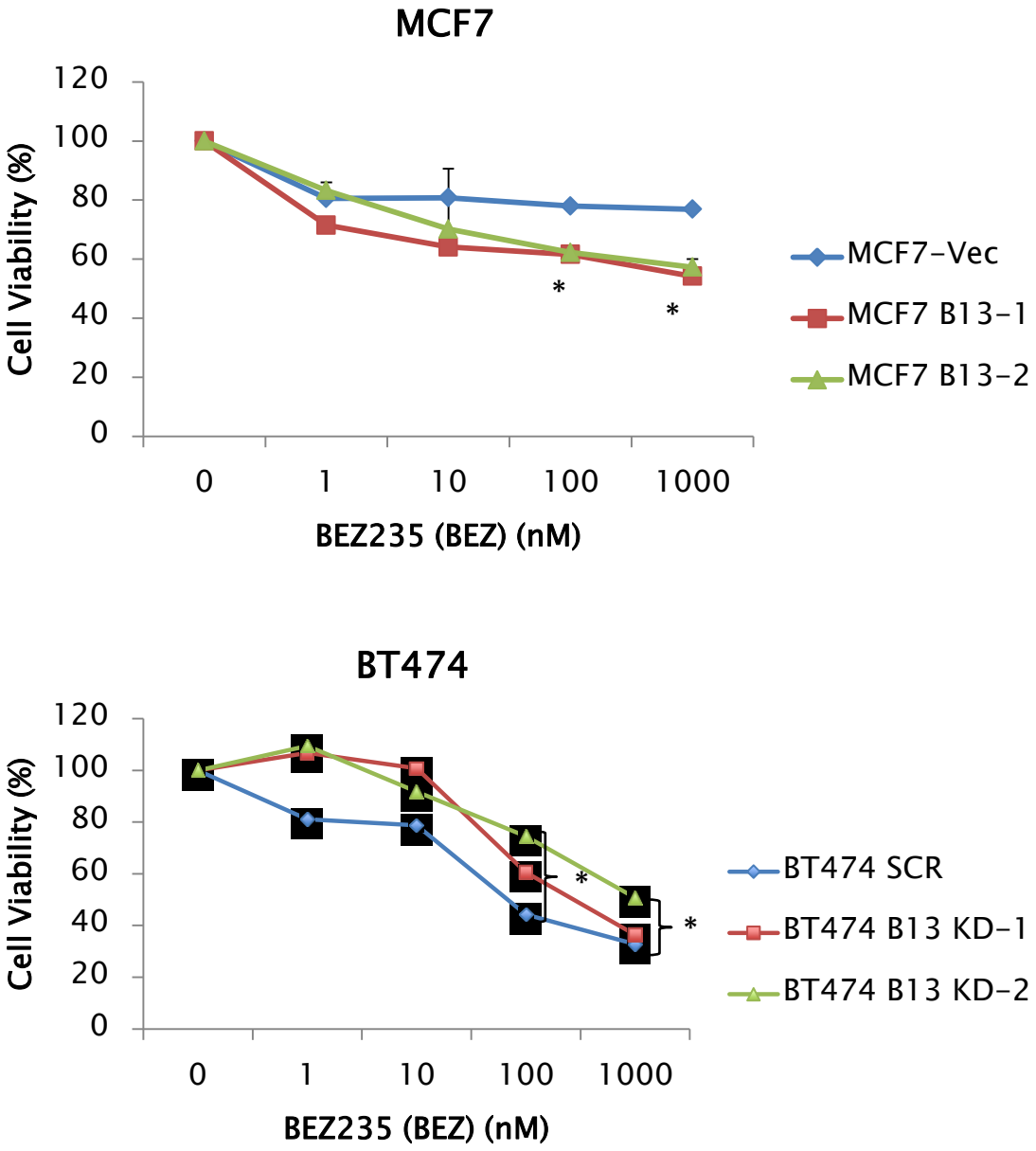

Figure 5.4. BEZ235 significantly inhibits viability of HOXB13-overexpressing cells in culture. MTT cell viability after treatment with increasing doses of BEZ in MCF7 HOXB13-overexpressing cells compared with MCF7 vector control and BT474-Scr cells compared with BT474 HOXB13-KD. (*, P<0.01)

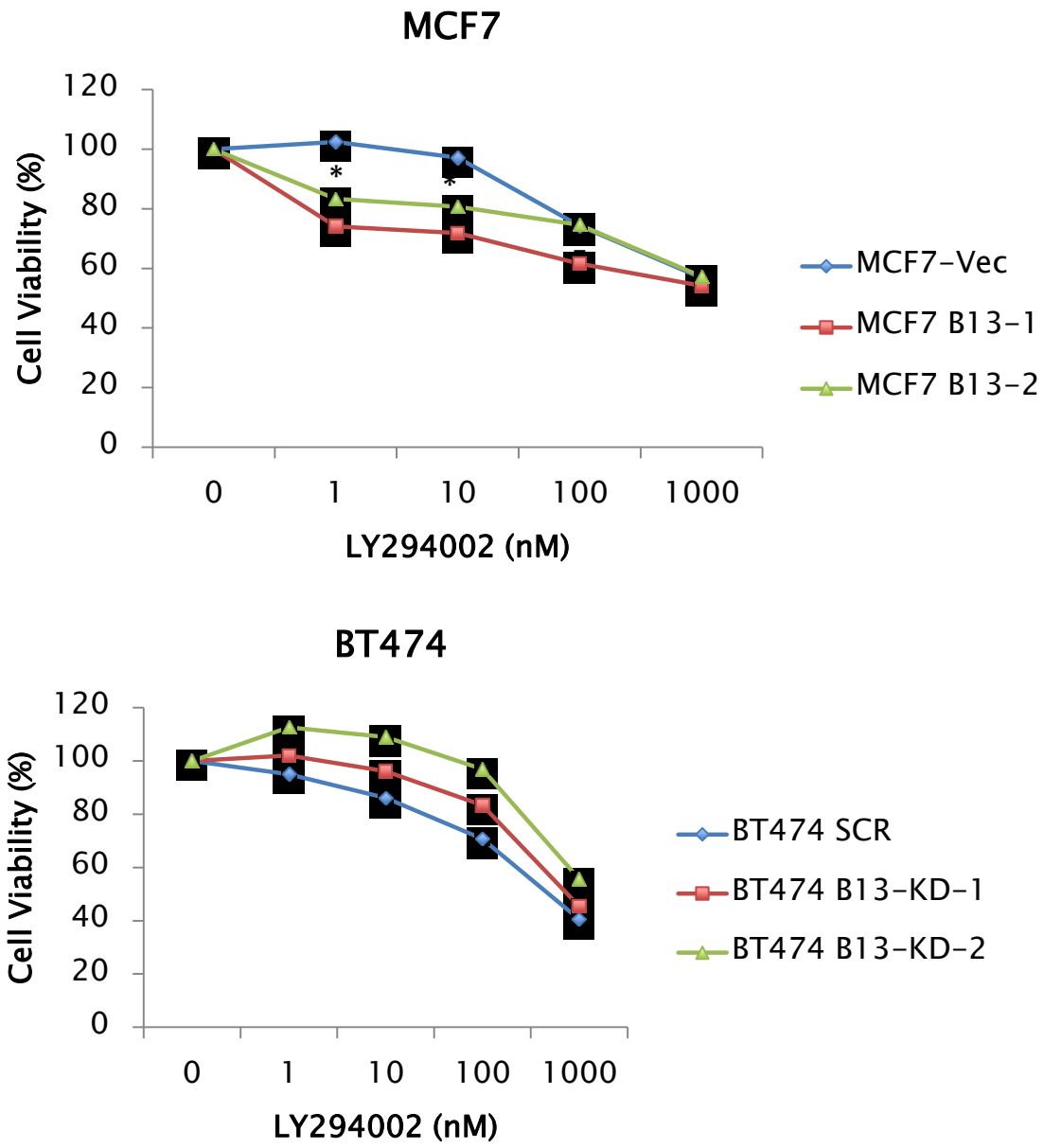

Figure 5.5. LY294002 does not significantly inhibit viability of HOXB13-overexpressing cells in culture. MTT cell viability after treatment with increasing doses of BEZ in MCF7 HOXB13-overexpressing cells compared with MCF7 vector control and BT474-Scr cells compared with BT474 HOXB13-KD. (*, P<0.05)

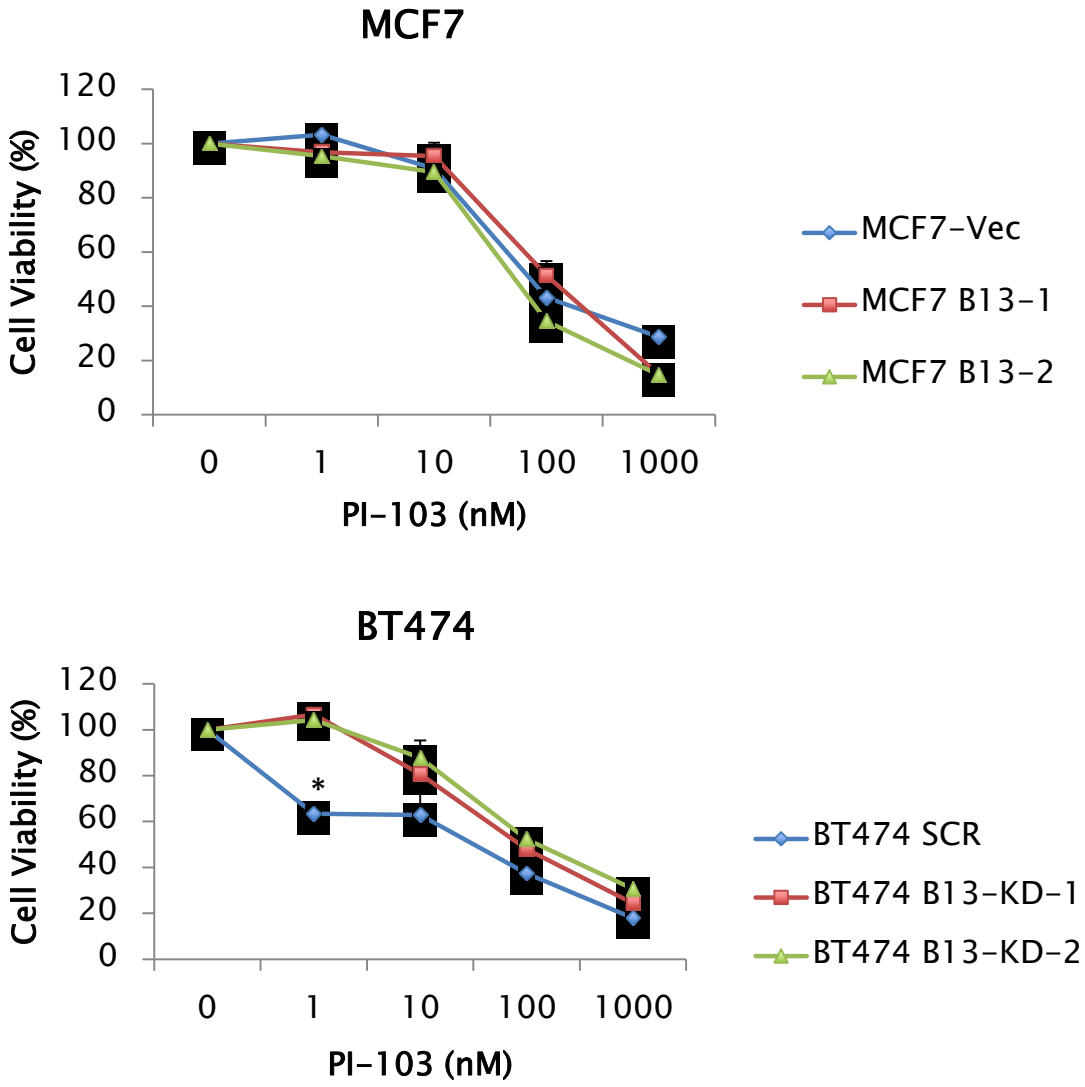

Figure 5.6. PI-103 does not significantly inhibit viability of HOXB13-overexpressing cells in culture. MTT cell viability after treatment with increasing doses of BEZ in MCF7 HOXB13-overexpressing cells compared with MCF7 vector control and BT474-Scr cells compared with BT474 HOXB13-KD. (*, $P<0.05$)

the MCF7 cell lines; however, this effect was not observed in the BT474 panel (Figure 5.5). PI-103 showed HOXB13-specific cell death at lower doses in BT474-Scr compared to BT474 HOXB13-KD cells, however; this effect was not replicated in our MCF7 HOXB13-overexpressing panel (Figure 5.6). Following these studies, we decided to move forward with BEZ235 to verify inhibition of PI3K signaling in the HOXB13-expressing cells by western blot analysis.

5.4 – NVP-BEZ-235 is effective at specifically inhibiting phospho-Akt activation in HOXB13-expressing cells

We wanted to confirm the inhibition of IL6-pathway downstream effectors such as PI3K/Akt upon BEZ treatment in our HOXB13-expressing cells via western blot. Due to the quick action of signaling molecules in the span of a few minutes to an hour, we employed 50 nM BEZ for 15-minute intervals up to an hour to determine its effects on both the overexpressing MCF7-HOXB13 and the BT474 HOXB13 KD cells. In this time course experiment, we observed a greater inhibition of phospho-Akt in the HOXB13-overexpressing MCF7 cells compared to vector, as well as in the BT474-Scr cells compared to the HOXB13 KD cells. This result correlates with our previous observations in the cell viability assays (65-67) showing HOXB13-specific BEZ effect and previous western blots showing higher levels of p-AKT in the HOXB13-expressing cell lines (Figure 3.5b).

Conclusions

As with rapamycin, we investigated other IL6 pathway effectors by testing inhibitors against these effectors in our HOXB13-expressing cell line models *in vitro*. We found TOC and BEZ to be ideal drug candidates that exhibit specificity against HOXB13-expressing cells. However, because BEZ has already been tested extensively in breast cancer (65-67) and newer drugs targeting the PI3K pathway are a subject of great interest in the field (68),

we decided to move forward to test TOC *in vivo*. Also, TOC has not been tested as a treatment for breast cancer and whose direct target is the pathway and molecule of interest.

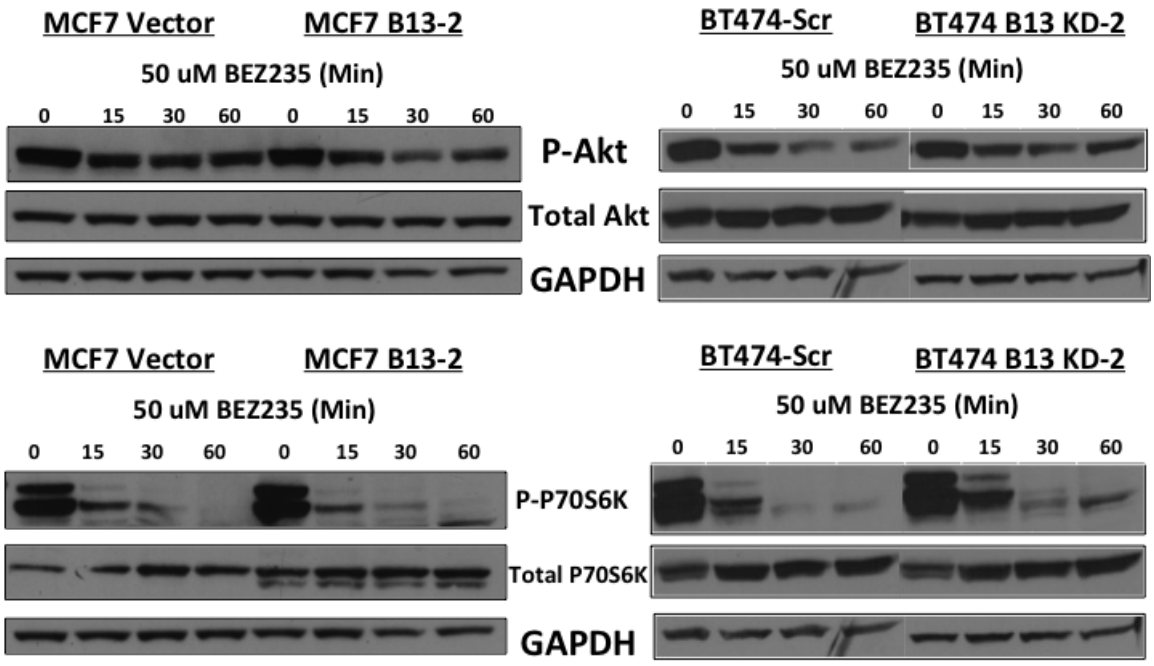

Figure 5.7. PI3K/Akt pathway activation in HOXB13-overexpressing cells via IL6 is inhibited by BEZ235 in a time-course manner. Western blot analysis of p-STAT3 (Y705), p-AKT (S473), p-p70S6K, and p-4EBP1 in MCF7 HOXB13 and BT474 HOXB13-KD cells compared with control cells treated with BEZ over time.

CHAPTER 6

In vivo targeting of the IL6 pathway in HOXB13-overexpressing breast cancer cell lines

6.1 – Tocilizumab (TOC) and BEZ235, both alone and in combination, inhibit HOXB13-overexpressing TAMR xenograft tumor growth

Based on the results of the Boyden chamber cell migration assay and the potent effects of TOC in this experiment, we embarked on studies with TOC to test inhibition of the IL6 pathway *in vivo*. However, because targeting two vulnerable pathways in order to achieve greater effect was an attractive option for treatment, and due to the high PI3K/Akt activity observed in the MCF7 HOXB13-overexpressing cells, we investigated TOC alone and in combination with BEZ on xenografts of our panel of cells to test both for an additive or synergistic effect. MCF7 HOXB13-overexpressing clone 2 xenografts were grown in NSG mice until tumors were palpable and then treated with vehicle control, TOC (100 μg i.p. 3 days per week), BEZ (30 mg/kg p.o. 5 days per week), or TOC and BEZ combined for 6 weeks. Although there were no changes in animal weight, BEZ-treated animals were noticeably more irritable. Our results showed that each drug alone and in combination inhibited HOXB13-overexpressing tumor growth (Figure 6.1). TOC alone had a marked effect, as did BEZ alone, however, the TOC and BEZ combination showed no improvement over BEZ alone, with no significant difference in tumor size in the TOC+BEZ groups.

Due to its extensive use in breast cancer and toxicity in both pre-clinical and clinical studies, we decided to focus on TOC alone for our further *in vivo* investigations. Because TOC was effective in inhibiting palpable HOXB13-overexpressing tumor growth, we tested the effect of TOC treatment on established 100mm cubed sized tumors. MCF7 HOXB13-

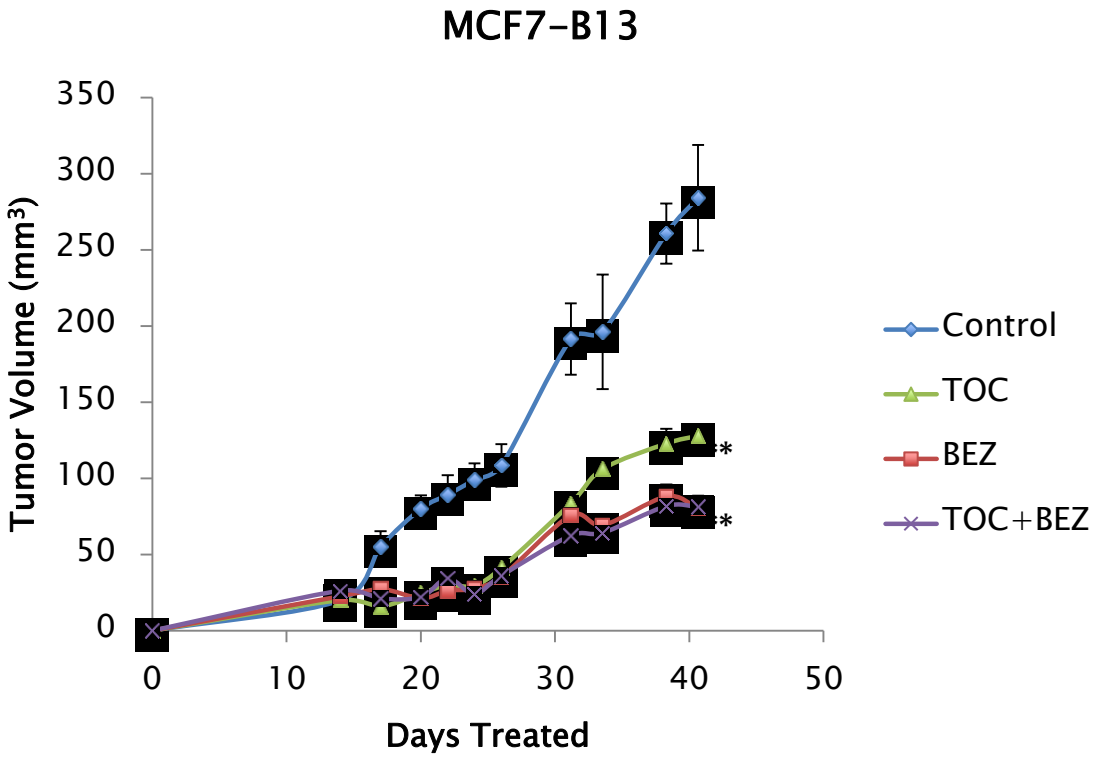

Figure 6.1. Treatment of MCF7 HOXB13-overexpressing xenografts with TOC and BEZ, both alone and in combination inhibits palpable tumor growth. Tumor growth of MCF7 HOXB13-overexpressing clone 2 cell subcutaneous xenografts in NSG mice treated with TOC and BEZ alone and in combination upon palpable tumor. (**, $P<0.005$)

tumors grew to 100 mm^3 and then treated with vehicle control or TOC (100 μg i.p. 3 days per week), with no changes in animal weight. We observed decreased tumor growth and regression upon TOC treatment compared to vehicle control (Figure 6.2), confirming the ability of TOC to inhibit HOXB13-overexpressing established-tumor growth.

To investigate the histological appearance of these tumors upon TOC treatment, we stained tumor sections with hematoxylin and eosin. MCF7-vector control xenografts from our previous studies grow as a dense, encapsulated structure with very little stroma. Sections from the xenografts from MCF7 HOXB13-overexpressing tumors were characterized by an increased stromal component, or desmoplastic response, seen as pink areas surrounding purple tumor cells, replicating the previously observed phenotype of the MCF7 HOXB13-overexpressing tumors. Upon TOC treatment, the increased stromal component was lost in the HOXB13-overexpressing tumors, showing inhibition of an increased stromal component and subsequent regression in HOXB13 tumor growth (Figure 6.3).

While our MCF7 HOXB13-overexpressing cell lines serve as a useful model to address the conversion of a TAM-sensitive cell line to a TAMR cell line through introduction and overexpression of a single gene like HOXB13, we decided to investigate the effect of TOC on our BT474-Scr cell line, due to its ER-positivity, endogenous high HOXB13 expression levels, and TAMR phenotype. Also, due to downregulation or abrogation of the IL6 pathway signaling via TOC treatment, we wanted to test if this action in our HOXB13-expressing cells could resensitize BT474-Scr to the effects of TAM. BT474-Scr xenografts were grown in NSG mice until tumors grew to 100 mm^3 and then treated with vehicle control, TOC alone (100 μg i.p. 3 days per week), TAM alone (500 μg in peanut oil for slow release, s.c. 5 days per week), or TOC in combination with TAM, with no changes in animal weight. TAM treatment did not affect HOXB13-expressing BT474-Scr

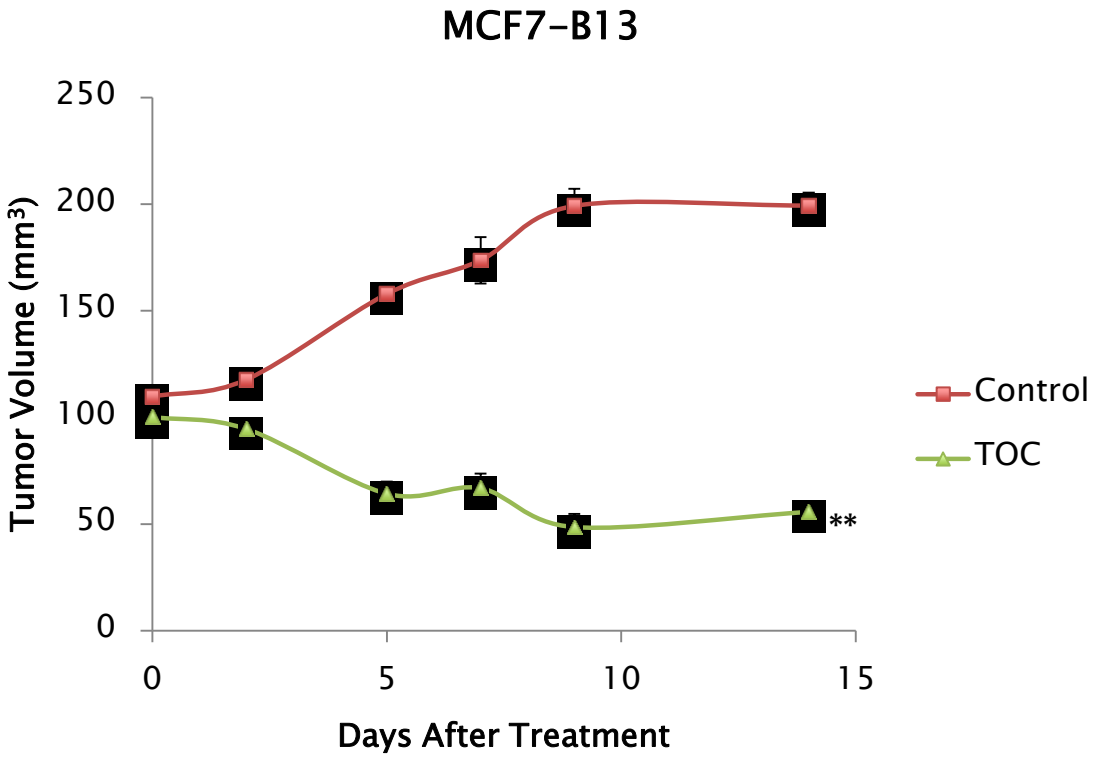

Figure 6.2. Treatment of MCF7 HOXB13-overexpressing xenografts with TOC inhibits established tumor growth. Tumor growth of MCF7 HOXB13-overexpressing clone 2 cell subcutaneous xenografts in NSG mice treated with vehicle control or TOC upon established tumor. (**, P<0.005)

tumor growth as seen in prior studies by our group and others that the cells are TAMR. TOC-treated mice showed a highly significant reduction in tumor growth compared to vehicle control alone or TAM alone. TOC in combination with TAM brought about even more significant reduction in tumor size; however, the difference between TOC alone and TOC+TAM was not statistically significant (Figure 6.4).

To confirm if BT474-Scr tumor regression due to TOC treatment was specific for the presence of high levels of HOXB13, we treated BT474 HOXB13-KD clone 2 cell line xenografts with TOC and observed that whereas BT474-Scr xenografts with high levels of HOXB13 were sensitive to TOC, knockdown of HOXB13 in BT474 renders the xenografts insensitive to TOC (Figure 6.5).

Conclusions

Our *in vivo* studies targeting the IL6 pathway showed TOC inhibition of HOXB13-expressing tumor growth in both palpable and established MCF7 HOXB13-overexpressing xenografts, inhibiting the stromal response characteristic of the cell line xenografts. We also observed tumor regression upon TOC treatment in established HOXB13-expressing BT474-Scr xenografts, however, HOXB13 knockdown in BT474 resulted in loss of response of the cells to TOC, suggesting a key role for high levels of HOXB13-expression in the IL6R-antibody-mediated therapeutic effect of TOC.

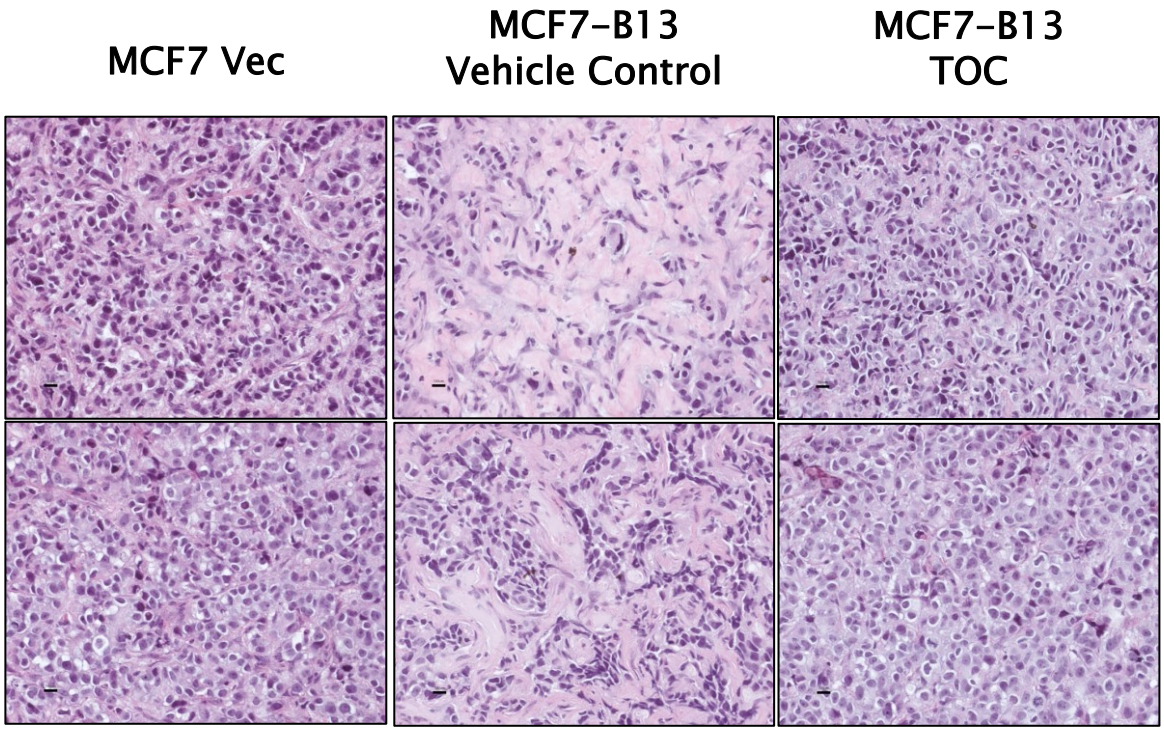

Figure 6.3. TOC treatment reversed stromal phenotype in MCF7 HOXB13-overexpressing xenografts. Hematoxylin and eosin-stained sections of MCF7 xenografts: MCF7-vector tumors grow as uniform sheets of cancer cells with little stromal component, whereas MCF7 HOXB13-overexpressing xenografts have significantly more stromal infiltration which is abrogated upon TOC treatment.

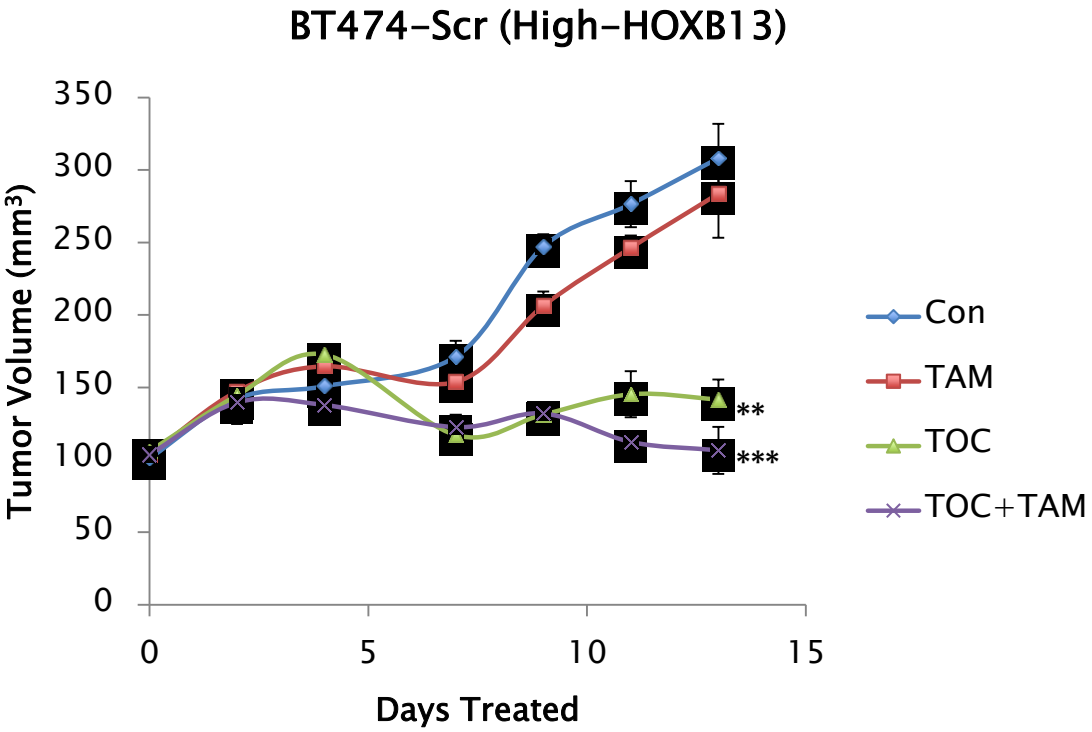

Figure 6.4. Treatment of HOXB13-expressing BT474-Scr xenografts with TOC alone, TAM alone, or in combination inhibits established tumor growth. Tumor growth of HOXB13-expressing BT474-Scr cell subcutaneous xenografts in NSG mice treated with vehicle control or TOC upon established tumor. (**, P<0.005)

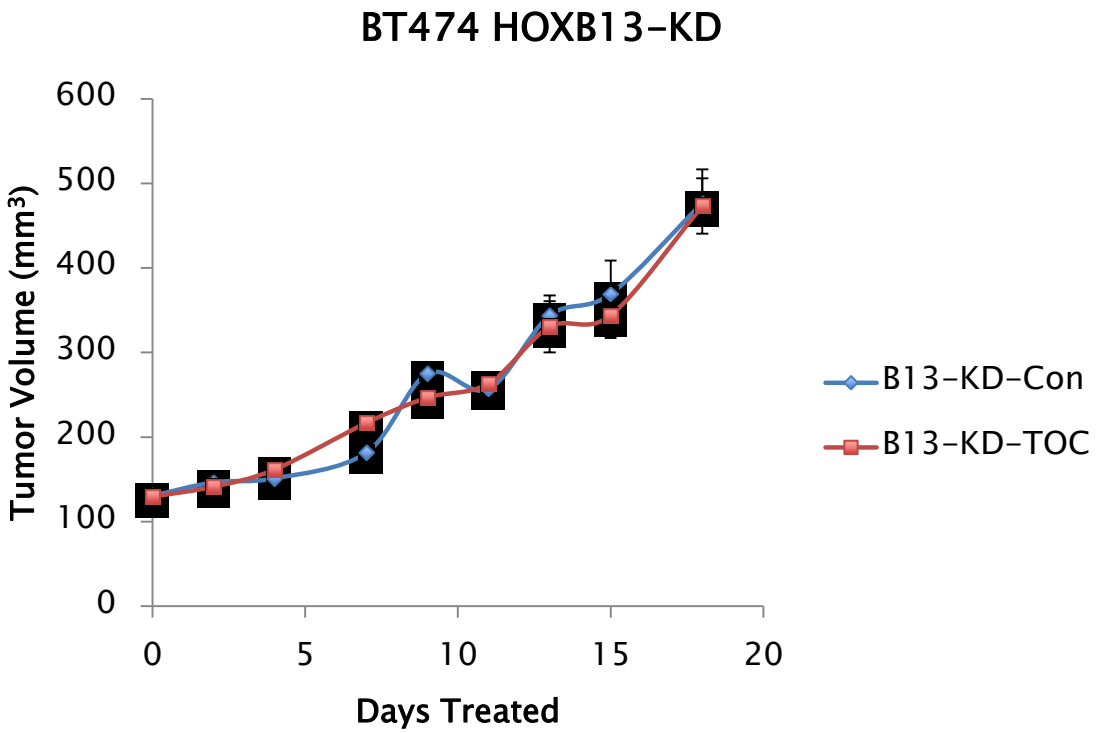

Figure 6.5. Treatment of HOXB13-knockdown BT474-HOXB13-KD xenografts with TOC does not inhibit established tumor growth. Tumor growth of HOXB13-knockdown BT474-HOXB13-KD cell subcutaneous xenografts in NSG mice treated with vehicle control or TOC upon established tumor. (NS)

CHAPTER 7

Discussion

Many facets of the molecular mechanisms underlying TAMR are known; of note, the predictive value of HOXB13 as a clinical biomarker of TAMR. In spite of this knowledge, for many years, no studies had examined the mechanisms by which HOXB13 contributed to disease aggression, specifically in TAM-R and metastatic recurrence. Thus, an evaluation of HOXB13 function in TAM-R breast cancer was critical to identifying new therapeutic targets. We conducted the first such examination of HOXB13 in breast cancer and showed its role in aggressive disease, consistent with clinical studies (33).

For the first time, we assessed HOXB13 in distant metastatic tumors and confirmed that tumors from non-survivors had significantly higher mRNA levels of HOXB13 compared with those from survivors, and HOXB13 expression significantly predicts disease recurrence and death from disease. We also showed that HOXB13 promoted proliferation and TAMR *in vivo*, consistent with the association of HOXB13 expression with metastatic disease recurrence in patients despite treatment with TAM. The experimental evidence and the increased stromal component in HOXB13-overexpressing tumor xenografts suggested HOXB13 promoted tumor formation and disease recurrence also by promoting other pathways, including proliferation and stromal recruitment.

An extensive body of literature has emerged that supports cross-talk and cooperation between stromal cells and tumor cells at multiple levels such as: the soluble factors elicited by tumor cells and those in response by the stromal cells, changes in the extracellular matrix, and alterations in cell adhesion, which collectively lead to a microenvironment conducive to tumor progression (69, 70). A major cell type that alters the tumor milieu is the stromal fibroblast. To study the notable stromal recruitment visible in HOXB13 overexpressing

tumors, and to decipher the role of IL6–mediated pathways in this function, we modeled the *in vivo* conditions in tissue culture. We showed the ability of normal murine and human fibroblasts to migrate toward conditioned medium from HOXB13-overexpressing tumor cells, but not from vector control cells; and that the action was abrogated by pre-treating the fibroblasts with anti-IL6R antibody. Although this approach replicated the recruitment of naïve fibroblasts to the tumor site by IL6 secreted by tumor cells, it would also be important to study, in the future, the molecular and biological differences between cancer-associated-fibroblasts (CAF) existing in the tumors and in normal fibroblasts in the breast, and how CAF action relates to hormone resistance mediated by HOXB13.

In our studies, we confirmed HOXB13-directed transcriptional activation of IL6, a common mediator of stromal recruitment IL6 is an activator of the AKT pathway, and that this activation is suppressed by mTOR inhibitors (55, 71). We specifically showed that the mTOR inhibitor rapamycin can suppress the proliferation of ER-positive, HOXB13-expressing cells *in vitro* and *in vivo*, alone or in combination with TAM. This work led us to conclude that HOXB13 expression makes the cells dependent on mTOR-mediated pathways for this proliferation. In spite of these results, these pathways needed to be more fully defined to identify other effective, potential mechanisms of HOXB13-mediated TAMR.

As with rapamycin in the aforementioned studies, further investigation for effective targeted treatment against HOXB13-mediated TAMR was conducted via microarray analysis of our BT474 HOXB13-KD cell line panel searching for novel molecules that correlate with HOXB13 expression. Further, we interrogated inhibitors against other effectors of the IL6 pathway such as IL6R, Stat3, and PI3K/Akt *in vitro* and *in vivo* against our HOXB13-expressing cell line panels.

We present the HOXB13-specific effects of the anti-IL6R antibody tocilizumab (TOC), a drug widely used to treat rheumatoid arthritis, but not yet considered as a treatment for breast cancer. Because the effects of rapamycin are published, we will discuss the effects of TOC alone. Our studies show that although TOC does not inhibit HOXB13-specific cell viability *in vitro*, it abrogates *in vitro* fibroblast migration to HOXB13-expressing conditioned media compared to vector and HOXB13-knockdown controls. Of note, our *in vivo* studies highlight the ability of TOC to inhibit both palpable and established tumor growth of both our MCF7 and BT474 HOXB13-expressing models, significantly reducing the stromal desmoplastic response typical of aggressive tumors (72) and characteristic of our HOXB13-overexpressing cell line model. Knockdown of HOXB13 in our BT474 cell line rendered xenografts insensitive to TOC treatment, thus confirming the specificity of TOC treatment against HOXB13-expressing cells. These results present tocilizumab as a promising drug candidate specifically targeting HOXB13-overexpressing TAMR breast cancer growth.

We addressed the importance of HOXB13-mediated overexpression of IL6 as a paracrine means of activating tumor associated stroma by establishing IL6-induced fibroblast migration and direct HOXB13 binding of the IL6 promoter; however, more studies are necessary to answer more specifics regarding this stroma-tumor interaction. A recent study has shown that cancer-associated fibroblasts (CAFs) can induce TAMR in the luminal (ER-positive) subtype of breast cancer via IL6 secretion and subsequent IL6 pathway signaling in the associated tumor cells (73), underlining the importance of the stromal component in TAMR. It would be interesting to investigate if HOXB13 may play a role in this model of TAMR, especially with regard to HOXB13 levels in the tumor cells before and after CAF

exposure, as our HOXB13 overexpressing cell line models are also characteristic of TAMR, stromal infiltration, and IL6 expression.

Our work provides evidence for the novel use of TOC for the treatment of ER-positive breast tumors with high levels of HOXB13 expression at initial diagnosis. Further pre-clinical *in vivo* experiments in other established TAMR or HOXB13-overexpressing cell lines in combination with TAM and/or AIs would be useful to confirm the specificity of TOC on HOXB13-expressing TAMR models. For the first time, TOC has been shown to inhibit *in vitro* and *in vivo* HOXB13-expressing tumor growth and thus is a promising candidate drug for the treatment of TAMR breast cancer.

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Mount St. Mary's College Dean's List, 2002-2006
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PUBLICATIONS

1. Shah N, Jin K, **Cruz LA**, Park S, Sadik H, Cho S, Goswami CP, Nakshatri H, Gupta R, Chang HY, Zhang Z, Cimino-Mathews A, Cope L, Umbricht C, Sukumar S. HOXB13 Mediates Tamoxifen Resistance and Invasiveness in Human Breast Cancer by Suppressing ERα and Inducing IL-6 Expression. **Cancer Res**. 2013 Sep 1;73(17):5449-58. doi: 10.1158/0008-5472.CAN-13-1178. Epub 2013 Jul 5.
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POSTERS AND ABSTRACTS

1. **LA Cruz**, K Jin, S Cho, and S Sukumar, Effective targeting and treatment of HOXB13-overexpressing tamoxifen-resistant breast cancer. Annual Safeway Breast Cancer Research Retreat, Baltimore, MD May 2014.
2. **LA Cruz**, K Huppi and JF Mushinski, miRNA found in the PVT1 locus enhances c-MYC expression and increases B-cell proliferation. National Institutes of Health Post-Baccalaureate Research Festival, Bethesda, MD May 2008.
3. **LA Cruz**, LY Romanova and JF Mushinski, Interactions of Miz-1 and Cytosolic c-Myc. National Institutes of Health Post-Baccalaureate Research Festival, Bethesda, MD May 2007.
4. **LA Cruz**, AJ Barbera and KM Kaye, Chromosome Binding of the Kaposi's Sarcoma-Associated Latency-Associated Nuclear Antigen. Annual Biomedical Research Conference for Minority Students, Atlanta, GA November 2005.
5. **LA Cruz**, MT Ocampo-Hafalla and GW Teebor, Characterization of Endonuclease III-Null and Endonuclease VIII-Null Mice. Annual Biomedical Research Conference for Minority Students, Dallas, TX November 2004.

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